INHIBITION PHENOTYPE SPECIFIC FOR *ori*λ REPLICATION-DEPENDENT PHAGE GROWTH, AND A REAPPRAISAL OF THE INFLUENCE OF λ *P* EXPRESSION ON *ESCHERICHIA COLI* CELL METABOLISM: P-INTERFERENCE PHENOTYPE

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ABSTRACT

Bacteriophage λ has been used as a model replicon system for forty years. While the basic λ replication initiation scheme has been elucidated for several decades, many aspects of the mechanisms are unclear. I wished to study two unanswered issues in λ replication initiation.

Replication initiation of *E. coli* and λ each depend upon a protein generally called a licensing factor, which brings the DnaB helicase protein to the origin site to begin DNA synthesis. The licensing factors are the products of host gene *dnaC* and λ gene *P*. The synthesis of P from λ DNA in an *E. coli* cell can competitively interfere with DnaC activity needed for *E. coli* replication initiation. I wished to learn more about what happens to a host cell when exposed to extended *P* expression. Previous studies in this laboratory suggested that i) the continuous expression of *P* was tolerated by a subset of exposed cells and that ii) host defects mapping to *dnaB* could suppress the effect of extended *P* expression (P-lethality). I used DNA sequencing to determine if these suppressor mutations were within *dnaB*. I screened known host mutations for their influence on P-lethality. In summary: *E. coli* strains with GrpD55 and GrpA80 defects were found to each have two point mutations within their *dnaB* genes. I was unable to isolate mutations within *P* that suppressed P-lethality and instead obtained regulatory mutations preventing wild type *P* expression. Two of these sequenced mutations showed that a *cI*[Ts] lambda repressor was reverted to *cI* wild type, blocking *P* expression at all assay temperatures. P-lethality was reversible in cells exposed to P for up to five hours, causing me to suggest that P-Interference be used in place of the term P-lethality. A non-inducible allele of *lexA* prevented P-mediated

cellular filamentation and enhanced P-Interference. This suggests that induction of the SOS response helps cells to tolerate extended *P* expression. A host strain containing a defective ClpXP protease significantly enhanced cellular sensitivity to P-Interference. This suggests an important role for the ClpXP chaperone-protease complex in degradation of P and cellular resistance to *P* expression. I present models to explain the P-Interference Phenotype.

Recent reports have re-opened the possibility that the t_O-*oop*-p_O element influences λ DNA replication initiation. I have also been investigating this possibility. I found that a plasmid with t_O-*oop*-p_O (the terminator, nucleotide sequence and promoter for OOP RNA) and *ori*λ DNA sequence was inhibitory to the development of *rep*λ phages, and designated this the Inhibition Phenotype (IP). In pursuing the mechanism for this inhibition, I mutated the t_O-*oop*-p_O and *ori*λ elements. I found that the expression of the 77nt OOP RNA transcript and the presence of four 18 base pair repeats (iterons) within *ori*λ were required for the IP. I isolated spontaneous phage mutants, resistant to the IP. I determined that singly infected cells were sensitive to the IP but that multiply infected cells escaped the IP. I propose that the IP to *rep*λ phage development is directed to the initial or theta mode of λ replication initiation. I found that the theta-mode of λ replication initiation can be bypassed, likely via recombination between multiple phage genomes within a singe cell. I propose models to explain the IP and also suggest a role for OOP RNA in the regulation of λ DNA replication.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

aa	amino acid
Amp	ampicillin
Att(P)	the site on the λ genome where site specific recombination occurs during phage integration into the host chromosome
ban	codes for bacteriophage P1 initiator protein Ban
BLA	background lowering activity
bp	base pair
c17	a CI-independent promoter able to transcribe genes *O* and *P*
cfu	colony forming units
ColE1	a plasmid where replication initiation is regulated by RNAI and RNAII interaction and Rop protein
cos	cohesive ends at either end of the λ vegetative genome; 12 bp ssDNA ends are important in genome circularization
dsDNA	double stranded DNA
EOP	efficiency of plating
g	gram
hsp	heat shock protein
imm	the immunity region; in λ composed of genes *cI, rexA and rexB* and the two operator sites, O_L and O_R
ice	proposed inceptor site
IP	the Inhibition Phenotype, the Inhibition Phenotype specific to *rep*λ phage development
IPTG	induces transcription from *lac* promoters
IS2	insertion sequence 2
ITN	iteron
Kb	kilo base pair
λ	bacteriophage lambda
λdv	a plasmid derived from λvir DNA; capable of autonomous replication; contains a λvir DNA fragment from *rexA* through *ren*
λvir	λ virulent phage; contains a v2 point mutation in o_L and v1v3 point mutations in o_R; operator mutations prevent CI from binding
mL	milliliter
moi	multiplicity of infection
NinR	this λ region between genes *P* and *Q* contains several recombination genes; e.g. *rap(ninG), orf146* and *ninB*
nt	nucleotide
O_L	leftward operator sequence – contains binding sites for Cro and CI and overlaps p_L
O_R	rightward operator sequence – contains binding sites for Cro and CI and overlaps p_R

O-some	the first nucleoprotein complex formed at *ori*λ, composed of O dimers bound to the four iterons of *ori*λ
OSA	OOP stimulating activity
*ori*λ	the lambda origin of DNA replication, comprised of four 18 bp iterons and 40 bp of AT rich DNA
*ori*C	*E. coli* origin of DNA replication, comprised of several DnaA boxes and a high AT rich region
P1	bacteriophage P1
P22	bacteriophage P22
Φ21	bacteriophage Φ21
Φ80	bacteriophage Φ80
434	bacteriophage 434
pfu	plaque forming units
phage	bacteriophage
π	mutations in λ gene *P* enabling λ to grow on GroP *E. coli* strains
p_{aQ}	λ promoter for antisense Q RNA production, regulated by CII
p_I	λ promoter for Int production, regulated by CII
p_L	λ leftward major promoter, regulated by CI and Cro
p_{lit1}	λ constitutive *rexB* promoter
p_{lit2}	λ rexB promoter; co-regulated with p_O promoter and *ori*λ-dependent replication initiation complex formation
p_O	λ promoter for OOP RNA, co-regulated with *ori*λ-dependent replication initiation complex formation
p_R	λ rightward major promoter, regulated by CI and Cro
p_{RE}	λ repressor establishment promoter, regulated by CII
p_{RM}	λ repressor maintenance promoter, regulated by CI and Cro
reg	mutations in λ gene *P* enabling λ to grow on Grp *E. coli* strains
*rep*λ	a phage containing the origin site and replication genes of λ
*rep*P22	a phage containing the origin site and replication genes of P22
18	P22 initiator gene; analogous to *O* from λ
12	P22 initiator gene; analogous to *P* from λ; also encodes helicase activity
orf48	P22 gene immediately before gene *18,* function unknown
repB	streptococcal plasmid pLS1 initiator gene; regulated by RNAII antisense RNA
repC	initiator gene for plasmid pT181; regulated by pT181 antisense RNA
Rex Exclusion	exclusion of T4*rII* mutant phage by a λ lysogen
ri^c	replication initiation constitutive mutants; produce CI-independent promoters to constitutively trancriptionally activate *ori*λ
RK	replicative killing phenotype; cryptic λ prophage trapped in chromosome initiates replication upon prophage induction; the event is lethal to the host cell
RNAI-RNAII	RNAII is a primer essential for ColE1 replication; RNAI is an

	antisense RNA responsible for regulating RNAI activity
rop	gene for the ColE1 plasmid Rop protein, which enhances RNAII's ability to inactivate RNAI
SD	Shine Delgarno site; ribosomal binding site
SIP	λ*cI*857 mutants that are able to **S**uppress the **I**nhibition **P**henotype
ssDNA	single stranded DNA
t_L	leftward termination site
t_R	rightward termination site
t_O-*oop*-p_O	the terminator, open reading frame and promoter for the 77 nt OOP RNA
TB	Tryptone Broth
ts	temperature sensitive
θ	the theta, circle-to-circle or early mode of λ replication
σ	the sigma, rolling circle or late mode of λ replication
wt	wild type

LAMBDA GENE PRODUCTS:

bet	gene for λ general recombination enzyme Bet; renatures complimentary SS DNA
cI	gene for the λ CI repressor; inhibits transcription from p_R and p_L; can inhibit or activate transcription from p_{RM}
cII	gene for the λ critical lysogenic regulator CII; activates transcription from p_E, p_I and p_{aQ}
cIII	gene for the λ CIII protein that stabilizes CII protein by inhibiting cellular FtsH/HflB degradation of CII
cro	gene for the λ Cro repressor that binds o_R and o_L to inhibit p_{RM} transcription; transiently induces transcription from p_L and p_R
exo	gene for the λ general recombination enzyme Exo; 5' to 3' exonuclease
gam	gene for the λ Gam protein that inhibits *E. coli* ExoV enzymatic activity; enhances the stability of concatameric DNA products
int	gene for the λ Int protein; integrates λ DNA into *E. coli* chromosome via site-specific recombination
N	gene for the λ early antiterminator, N; binds to *nutR* and *nutL* sites on λ mRNA, required for bypass of t_{L1}, t_{R1} and t_{R2} transcriptional terminators
O	gene for the λ initiator protein O; binds to iteron sites of *ori*λ and binds P
P	gene for the λ initiator protein P; brings DnaB to *ori*λ-O complex; interacts with ssDNA, O, DnaB, DnaJ, DnaK, GrpE and DnaA
Q	gene for the λ late antiterminator protein Q; allows transcription to pass through $t_{R'}$

ren	λ gene, function unknown, thought to allow λ to escape Rex Exclusion
rexA-rexB	λ genes that confer exclusion of T4*rII* mutant phage
xis	gene for the λ Xis protein; works with Int to exise the prophage from the chromosome

E. COLI GENE PRODUCTS:

clpA	gene for the Hsp100 chaperone; interacts with ClpP to form ClpPA protease
clpB	gene for the Hsp100 chaperone involved in the unfolding of damaged proteins; may interact with ClpP
clpP	gene for the Hsp100 protease; requires complexing with ClpA or ClpX for substrate recognition
clpX	gene for the Hsp100 chaperone; interacts with ClpP to form ClpPX protease
crp	gene for the catabolite regulatory protein is involved in catabolite repression; regulated by the Tic antisense RNA
dnaA	gene for the *E. coli* initiator protein; transcriptional activator
dnaB	gene for the *E. coli* replicative DNA helicase
dnaC	gene for the *E. coli* initiator protein; DnaB loading protein
dnaG	gene for primase; lays down RNA primers which are extended by DNA polymerase III
dnaJ	gene for the DnaJ heat shock protein, chaperone; refolds misfolded proteins; dissociates protein aggregates; removes P from the λ preprimosomal complex
dnaK	gene for the DnaK heat shock protein, chaperone; refolds misfolded proteins; dissociates protein aggregates; removes P from the λ preprimosomal complex
DNA polymerase III	*E. coli* replicative polymerase for synthesizing DNA; the holoenzyme is a complex heteromultimer containing many subunits
RNA polymerase	required to transcribe mRNA; the holoenzyme is a complex multimer containing many subunits
dsrA	gene for DsrA; an antisense RNA capable of regulating *hns* and *rpoS* expression
ExoV	encoded by *recBCD* genes; an exonuclease that degrades linear dsDNA
groP	alleles of *dnaB* that are functional for *E. coli* replication at all temperatures, but are non-functional for λ replication at 42°C; can be suppressed by π mutations in gene *P*
grpA80	an allele of *dnaB* that is functional for *E. coli* replication at all temperatures, but is non-functional for λ replication at 42°C; can be suppressed by π or reg mutations in gene *P*
grpD55	an allele of *dnaB* that is functional for *E. coli* replication at all

	temperatures, but is non-functional for λ replication at 42°C; can be suppressed by π or reg mutations in gene *P*
grpE	gene for the heat shock protein GrpE; chaperone, refolds misfolded proteins; dissociates protein aggregates; removes P from the λ preprimosomal complex
hflA	gene for Hsp70(*hflC-K);* modulates the activity of the essential protease HflB
hflB	gene for the HflB cellular protease; also called FtsH; degrades CII
hns	gene for the major nucleoid protein H-NS; global transcription factor
hup	gene for the HU nucleoid protein; constrains supercoils
lexA	gene for the LexA SOS repressor
lig	gene for DNA ligase; seals DNA nicks
lon	gene for the ATP-dependent cytoplasmic protease Lon
micF	gene for the antisense RNA molecule MicF; regulates *ompF* expression
mutH	gene for the MutH component of the mismatch repair system; binds hemimethylated GATC sequences and causes a nick
ompF	gene for OmpF; outer membrane protein
oxyS	gene for OxyS; an antisense RNA capable of regulating up to 40 *E. coli* genes
polI	gene for DNA polymerase I
pcnB	gene for PAPI; polyadenylates RNA
recA	gene for RecA; essential recombination gene; DNA pairing and strand exchange; RecA binding to ssDNA induces the SOS response
recB	gene for RecB; component of *recBCD* ExoV enzyme which degrades linear dsDNA
recC	gene for RecC; component of *recBCD* ExoV enzyme which degrades linear dsDNA
recD	gene for RecD; component of *recBCD* ExoV enzyme which degrades linear dsDNA
recF	gene for RecF; RecF recombination pathway is important in the formation of plasmid linear multimers
rnc	gene for RNase III; degrades dsRNA molecules
rpoH	gene for σ^{32}; heat shock sigma factor, allows RNA polymerase to recognize heat shock promoters
rpoS	gene for σ^{s}; stationary phase sigma factor; allows RNA polymerase to recognize stationary phase promoters
seqA	gene for SeqA; proposed to be involved in regulating transcription from p_R
ssb	gene for the Single-stranded DNA binding protein (SSB)
ssrA	gene for 10Sa RNA; tags peptides stalled in translation with the 11 aa degradation signal AANDENYALAA

CHAPTER 1. INTRODUCTION

1.1. BACTERIOPHAGE λ LIFESTYLE CHOICES

λ, a well characterized temperate bacteriophage, has been used as a model system to study many molecular processes including transcription, recombination and DNA replication (as reviewed in Wegrzyn and Wegrzyn, 2005; Oppenheim *et al,* 2005; Stahl, 1998).

λ belongs to a large family of temperate bacteriophages termed lambdoid phages, which share many key traits and can be characterized by six main features (Campbell, 1996). Lambdoid phages are temperate, *i.e.* upon the infection of a host cell, they make a choice between one of two developmental pathways; they integrate their dsDNA into the host cell chromosome via site specific recombination; they use a positive mode of operon control, *i.e.* antitermination; the early lytic functions are organized into divergently transcribed operons; the operons are regulated by a single repressor specified by a gene located between the two operons; and they share a common arrangement of the genome into functional modules capable of being exchanged within the family to produce fully functional recombinants (Brussow and Hendrix, 2002; Hendrix *et al,* 1999). A simplified λ vegetative map is shown in Fig. 1.1, demonstrating the organization of the phage genome into functional modules. All lambdoid phages contain a similar structural layout of genes with common regulatory

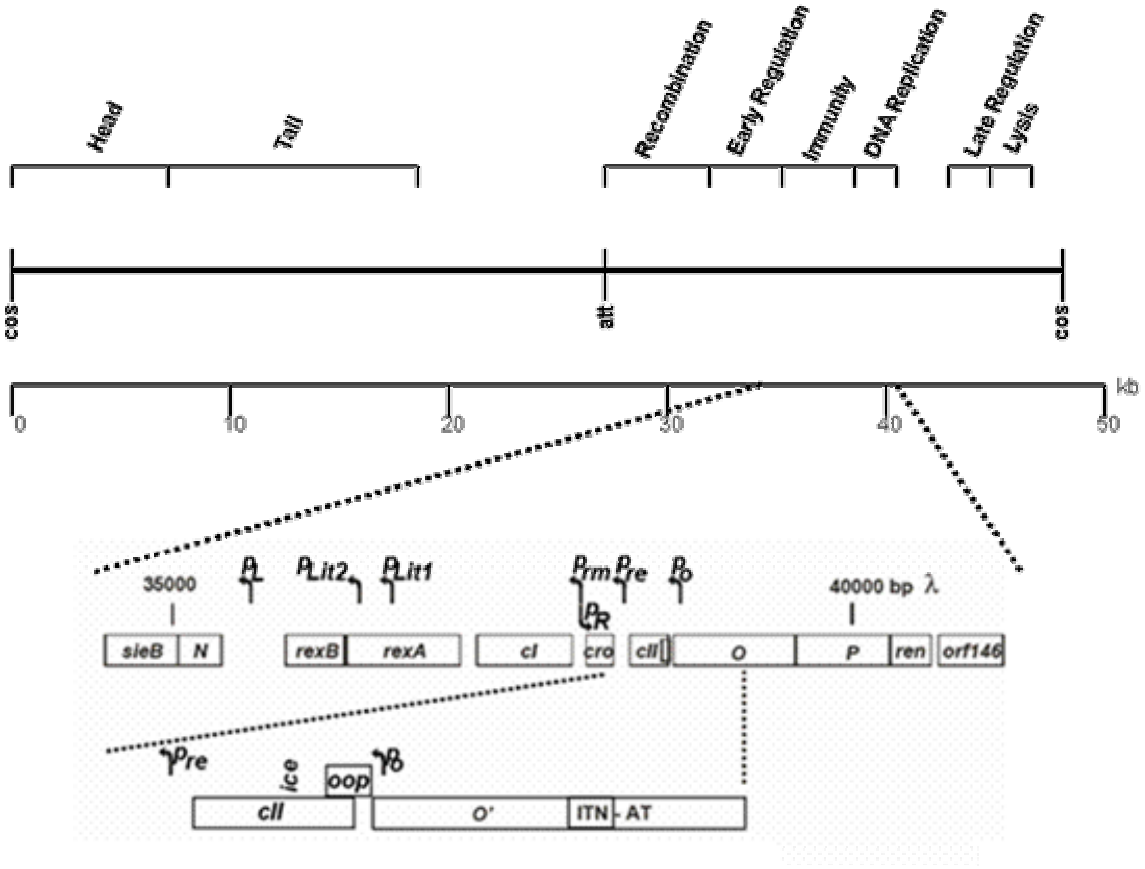

Figure 1.1. Bacteriophage λ Vegetative Map. The 48502 base pair (bp) λ genome is presented in the vegetative form (from *cos* site to *cos* site). The genome is expanded from approximately 35000-45000 to highlight the early regulation to DNA replication regions, with the important promoters being indicated. The *cII* and *O* genes are further amplified in order to demonstrate the relative position of the λ origin of replication, *ori*λ (ITN-AT), and the overlap between the *cII* and *oop* genes.

patterns.

Upon infecting a susceptible *E. coli* host cell, λ can undergo one of two possible developmental pathways, *i.e.* the lytic or lysogenic pathways. The decision to undergo lysogeny or lysis depends on environmental factors (*e.g.* temperature or multiplicity of infection) and on host cell factors, *i.e.* the actual physiological state of the cell (Kobiler *et al,* 2005).

1.1.1. Lysogeny vs. Lysis

λ DNA enters the cell in a double stranded linear form (from *cos*-*cos* arrangement as demonstrated in Fig 1.1). The DNA immediately circularizes through pairing and ligation of the complimentary 12 bp *cos* sequences at either end of the vegetative genome (Hershey *et al,* 1963). Transcription occurs from the phage promoters p_L and p_R to produce protein products N and Cro, respectively, Fig. 1.1. The N protein allows transcription to continue past terminators t_{L1} and t_{R1}, producing protein products CIII and CII, respectively. The CII protein has been described as being the "critical lysogenic regulator" (Oppenheim *et al,* 2005) as its activity is critical in initiating the lysogenic course. CII is very unstable, and CIII increases its stability by inhibiting the degradation of CII by a host protease (Hoyt *et al,* 1982; Altuvia and Oppenheim, 1986; Kihara *et al,* 1997). It was originally suggested that HflA was responsible for the degradation of CII as *hflA*-defective cells showed increased CII levels and increased frequency of lysogenization compared to wild type cells (Hoyt *et al,* 1982). It was later demonstrated that HflB was the cellular protease responsible for CII degradation (Kihara *et al,* 1997; Shotland *et al,* 2000); HflA is

important in modulating HflB levels (Kihara *et al,* 1997). CII binds to and activates the p_{RE} repressor establishment promoter, producing CI repressor protein (Echols and Green, 1971; Katzir *et al,* 1976; Shimatake and Rosenberg, 1981). CII also activates transcription from p_I to produce Int protein, which is required for the integration of the phage genome into the host chromosome (Echols and Green, 1971; Shimatake and Rosenberg, 1981). Finally, CII activates transcription from the p_{aQ} promoter, involved in the antisense regulation of the late antiterminator protein Q (Rattray *et al,* 1984).

A molecular competition occurs between the Cro and CI repressor proteins, which bind to the same DNA operator sites, O_L and O_R. The operator sites are very important, as their occupancy levels control further transcription from the promoters p_L and p_R (Ptashne *et al,* 1980). In essence, in directing a phage towards lysis, Cro prevents transcription from p_{RM}, blocking the maintenance of CI transcription. If Cro out-competes CI for binding to the operator sites, transcription from p_L and p_R is transiently permitted, and the lytic cycle develops (Ptashne *et al,* 1980; Kobiler *et al,* 2005). At high concentrations, the weak Cro repressor partially inhibits transcription from p_L and p_R, decreasing early gene expression and allowing the expression of late genes to occur. During the lytic cycle, nearly all phage genes are transcribed, and DNA replication and phage morphogenesis occur, ultimately leading to cell lysis and the release of mature phage particles. If CI out-competes Cro for binding to the operator sites, transcription from p_L and p_R is blocked, and transcription from the p_{RM} repressor maintenance promoter is allowed (producing more CI protein), permitting the lysogenic cycle to develop (Ptashne *et al,* 1980). Transcription from the weak p_{RM} promoter is increased 10-fold by the activity of CI (Michaelowski and Little, 2005). It

has recently been shown that if the p_{RM} promoter contains a mutation which increases its basal level of transcription, CI-mediated positive regulation is not required to promote and maintain the lysogenic state (Michaelowski and Little, 2005). High levels of CI inhibit transcription from p_{RM} (Ptashne *et al,* 1980) in an autoregulatory loop. During lysogeny, most phage genes (except *cI, rexA* and *rexB*) are repressed and the phage genome is integrated into the genome of the host cell, via Int protein activity. The lysogenic state is extremely stable, with prophage induction occurring very rarely and only under specific circumstances, such as host cell DNA damage.

If the host cell undergoes DNA damage, the induction of the cellular SOS response causes the λ prophage to enter the lytic pathway of development. CI repressor undergoes autocleavage and falls off of the O_L and O_R regulatory sites. Cro protein binds to O_R, preventing production of further CI protein from the p_{RM} promoter. Transcription from both p_L and p_R begins the lytic process. Int and Xis proteins are produced from p_L, allowing the excision of the prophage, which then circularizes, leading to lytic development. As Cro levels increase, transcription from both p_L and p_R is inhibited, leading to repression of the *cII* gene. As CII levels are low, p_E, p_I and p_{aQ} promoters remain inactive, inhibiting the lysogenization pathway. It was believed that the role of Cro in prophage induction was primarily to repress p_{RM} transcription to inhibit CI production (Ptashne *et al,* 1980). Recent studies indicate that the regulation of p_{RM} during prophage induction may be a secondary Cro function, less important than the down regulation of CII levels (Svenningsen *et al,* 2005; Oppenheim *et al,* 2005).

1.1.2. Passive vs. Active DNA Replication

In the lysogenic developmental pathway, the phage genome (now called a prophage) is integrated within the *E. coli* host cell chromosome between the galactose and biotin operons. As the phage replication genes are not expressed in this state, the λ genome is unable to replicate autonomously. However, every time the host cell replicates its chromosome, the λ genome is replicated passively.

In the lytic developmental pathway, the λ genome does not become integrated within the host genome, remaining as a circular, negatively supercoiled element, capable of autonomous replication. In this case, λ replicates from its own origin of replication, *ori*λ, requiring several host and phage gene products.

1.1.3. The Replicon Model

The Replicon Model was proposed by Jacob *et al,* in 1963 as a way to explain how DNA replication initiation might be regulated. A replicon was defined as "the fundamental genetic unit for autonomous replication and self-regulation" (Jacob *et al,* 1963). A replicon, as proposed in the model, is comprised of two essential elements, a *cis*-acting replicator site and a *trans*-acting replicator protein. Bacteriophage λ is a well studied model replicon. The λ replicator site, *ori*λ, is the site where autonomous phage DNA replication originates. λ encodes two *trans*-acting replicator proteins, O and P (Campbell, 1961; Joyner *et al,* 1966; Ogawa and Tomizawa, 1968), which act at *ori*λ to promote DNA replication initiation (Takahashi, 1975b).

λ has two modes of DNA replication, θ (theta or circle to circle) and σ (sigma or rolling circle) (Kiger and Sinsheimer, 1969). θ replication is the initial type of

replication seen from *ori*λ, as described by the Replicon Model. After several rounds of θ replication (Carter and Smith, 1970), about 15 minutes after infection, λ abruptly switches to the σ mode of replication (Enquist and Skalka, 1973; Bastia *et al,* 1975; Klinkert and Klein, 1978). The products of σ replication are concatemers (Smith and Skalka, 1966; Tomizawa and Ogawa, 1968; Eisen *et al,* 1968; Skalka, 1971), which are the preferred templates for the packaging of λ DNA into phage heads (Salzman and Weissbach, 1967; Salzman and Weissbach, 1968; Feiss and Becker, 1983).

1.2. THE θ MODE OF λ DNA REPLICATION

θ DNA replication initiation from *ori*λ develops as the result of a complex series of events occurring at *ori*λ. A discrete, well characterized series of specialized nucleoprotein complexes, involving both phage and host elements, develop at *ori*λ as a precursor to actual DNA synthesis. This series of events will be discussed in greater detail in the following sections.

1.2.1. Elements Comprising *ori*λ

*Ori*λ, the *cis*-acting replicator site for λ θ DNA replication, is comprised of two major elements, the iterons (ITNs) and the AT rich region (Scherer, 1978; Moore *et al,* 1979). *Ori*λ contains four 18 bp inverted repeats, each of which is a dyad of hyphenated symmetry, known as an iteron (Scherer, 1978; Moore *et al,* 1979). Periodic tracts of adenine residues cause the iteron domain of *ori*λ to assume a bent character (Zahn and Blattner, 1985). The iterons are directly followed by 40 bp of AT rich DNA, with a high pyrimidine content on one DNA strand, and a high purine

content on the other (Grosschedl and Hobom, 1979), likely enhancing the intrinsically curved structure of *ori*λ. *In vitro* deletion mapping studies have demonstrated that the minimal functional *ori*λ consists of at least two iterons and at least 35-40 nt of the adjacent AT rich region (Wickner and McKenny, 1987; Dodson *et al,* 1989). Initially, genetic mapping suggested that *ori*λ was located just to the left of gene *O* (Stevens *et al,* 1971). Later results more accurately mapped *ori*λ to be within the coding sequence for *O* (Furth *et al,* 1977; Denniston-Thompson *et al,* 1977). Furth *et al.* (1977) genetically mapped four *ori*λ mutations, r93, r96, r99 (isolated by Rambach, 1973) and *ti*12 (isolated by Dove *et al,* 1971) to *O*, and Denniston-Thompson *et al.* (1977) sequenced the mutants, directly demonstrating mutated *ori*λ sequences within gene *O*.

1.2.2. Transcription from p_R to produce O and P Initiator Proteins

Transcription from the promoter p_R, regulated by the CI repressor protein, produces the λ gene products O and P (Campbell, 1961; Brooks, 1965; Eisen *et al,* 1968; Brachet *et al,* 1970), the initiator proteins required for *ori*λ-dependent θ DNA replication (Joyner *et al,* 1966; Eisen *et al,* 1968; Kumar and Szybalski, 1970; Matsubara, 1976; Enquist and Skalka, 1978; Tsurimoto and Matsubara, 1982; Tsurimoto *et al,* 1982).

1.2.3. The "O-some"

1.2.3.1. The λ O Protein

The λ *O* gene encodes a 299 amino acid (aa) protein (Scherer, 1978). The N-

terminal region contains a DNA binding domain (Furth *et al,* 1977; Furth and Yates, 1978; Wickner and Zahn, 1986), allowing O to bind to the iteron sequences of *ori*λ (Tsurimoto and Matsubara, 1981). The O protein interacts with the λ P protein (Tomizawa, 1971) and it has been suggested that the C-terminal region of O contains a P-binding domain (Furth *et al,* 1977; Furth and Yates, 1978; Wickner and Zahn, 1986). The two functional domains are separated by a flexible linker region (Gonciarz-Swiatek *et al,* 1999). O has a very short half life *in vivo* (1.5 minutes) (Wyatt and Inokuchi, 1974; Wegrzyn *et al,* 1992), due to degradation by the ATP-dependent host protease ClpXP (Bejarano *et al,* 1993; Zylicz *et al,* 1998; Gonciarz-Swiatek *et al,* 1999).

1.2.3.2. Formation of the "O-some"

The first nucleoprotein complex to develop is the "O-some" (Dodson *et al,* 1985). Tsurimoto and Matsubara (1981) were the first to directly demonstrate the binding of O protein to the ITN sites of *ori*λ. They showed that each iteron contains two potential O binding sites (Tsurimoto and Matsubara, 1981). They showed that O protein binds to each iteron as a dimer, with each O monomer binding to a half site. The presence of four iterons allows the binding of at least four O dimers to occur (Tsurimoto and Matsubara, 1981). Experimental evidence showed that the minimum molar ratio of O protein to *ori*λ DNA required for substantial DNA unwinding is 20:1 (Dodson *et al,* 1986), suggesting that more than eight O monomers may be present in a functional O-some. ITN-2 and ITN-3 are bound by O first, followed by binding to ITN-1 and ITN-4 at higher O concentrations (Tsurimoto and Matsubara, 1981).

However, the O dimers are capable of further interaction; each O dimer bound to an iteron is able to interact with another (Schnos *et al,* 1989). The binding of O protein to ITN-2 and ITN-3 is proposed to bend the DNA sufficiently so that O binding at the outer sites (*i.e.* ITN-1 and ITN-4) can close the O-some loop via protein-protein interactions. This combination of DNA-protein and protein-protein interactions produces a large mass of O protein in the center, with the iteron DNA wrapped along the outer edges. This large O-ITN mass is referred to as the "O-some" (Dodson *et al,* 1985; Dodson *et al,* 1986; Alfano and McMacken, 1989A). The large distortion of the DNA structure caused by the O-some formation produces torsional stress on the adjacent AT rich region, causing the dsDNA to become slightly destabilized and partially unwound, as demonstrated by the presence of S1-sensitive and *Eco*RI-resistant ssDNA (Schnos *et al,* 1988; Alfano and McMacken, 1988). The S1-sensitive region extends from ITN-4 through the entire AT rich region, with approximately 23-49 bp of DNA being unwound (Schnos *et al,* 1988). The deletion of the AT rich region of *ori*λ abolishes DNA unwinding (*i.e.* active DNA synthesis), but has no effect on the formation of the O-some (Dodson *et al,* 1986; Wickner and McKenny, 1987).

1.2.4. P-DnaB Interaction

1.2.4.1. The λ P Protein

The λ P protein is the second phage-encoded initiator protein. Unlike the O protein, the 233 aa P protein is very stable, with a half life of up to an hour (Wyatt and

Inokuchi, 1974; Miwa *et al,* 1983). The N-terminal portion of P is assumed to contain an O binding domain (Reiser *et al,* 1983), while the C-terminal domain is involved with host protein interaction, specifically interaction with the host DnaB replicative helicase (Wickner, 1978; Reiser *et al,* 1983). Unlike the O initiator protein, P was not shown to directly interact with *ori*λ DNA (Tsurimo and Matsubara, 1982; Tsurimoto *et al,* 1982). However, more recently (Learn *et al,* 1997), P has been shown to interact with ssDNA in the vicinity of the origin site. The role of P in λ replication initiation is to bridge the gap between *ori*λ and the required host encoded replication proteins. Specifically, the major function of P is to bring the host DnaB helicase to the λ origin of replication. P is capable of interacting with the λ O protein (Tomizawa, 1971) and with several host proteins, including DnaA (Wegrzyn *et al,* 1996; Datta *et al,* 2005a; Datta *et al,* 2005b), DnaB (Georgopoulos and Herskowitz, 1971; Saito and Uchida, 1977; Klein *et al,* 1980), DnaJ (Yochem *et al,* 1978), DnaK (Yochem *et al,* 1978), GrpE (Zylicz *et al,* 1987) and possibly with RNA polymerase (McKinney and Wechsler, 1983).

Conditional *E. coli* mutants have been isolated that prevent λ replication initiation but still function for cellular replication. Such mutations have been localized to host genes *dnaB, dnaJ, dnaK* and *grpE* (Georgopoulos and Herskowitz, 1971; Saito and Uchida, 1977). Some of these λ replication blocks are suppressible by mutations in the λ gene *P*, which have been called π or *reg* mutations. All fourteen sequenced π mutations contain single point mutations within the C-terminal half of *P* (Reiser *et al,* 1983). Interestingly, regardless of which host strain the π mutants were isolated on, most π mutants were able to grow on any host strain defective for λ replication

initiation (Konieczny and Marszalek, 1995). For example, the πA66 mutant, which was originally isolated on the host GroPA15 strain, harboring a defect in *dnaB* (Georgopoulos and Herskowitz, 1971), was able to replicate and form plaques on *dnaB, dnaK, dnaJ* or *grpE* host strains defective for λ replication (Konieczny and Marszalek, 1995) or even a *dnaA*46 host strain defective for λdv plasmid replication (Wegrzyn *et al,* 1996). It has been suggested that the π phenotype confers to these P mutants a weak binding affinity to DnaB (Konieczny and Marszalek, 1995).

Early plasmid studies indicated that high levels of P protein were deleterious to host cells. In 1979, Klinkert and Klein overproduced P protein using a *lacI*/IPTG/*lacZ* promoter system. They suggested that high levels of P protein inhibited host DNA replication at the stage of initiation. They showed that high levels of P protein inhibited cell division, increasing the frequency of filamentation. An important finding by Klinkert and Klein was that high P levels did not cause host cell killing. Tsurimoto *et al.* (1982) over-expressed the λ *O* and *P* genes from tandem p_L and p_R promoters at 42°C. The results shown by Tsurimoto *et al*. (1982) were slightly different than those seen by Klinkert and Klein. Tsurimoto *et al*. demonstrated an immediate cessation of cellular DNA synthesis, gradual cell killing and no filament formation when O and P were simultaneously over-expressed. Both groups hypothesized that P was sequestering host DnaB protein and interfering with host cell DNA synthesis (Klinkert and Klein, 1979; Tsurimoto *et al,* 1982). In 1991, Maiti *et al*. demonstrated that high levels of P protein constitutively over-expressed from the p_R promoter (simultaneously with genes *cro, cII, O* and *ren*) caused host cell killing (Maiti *et al,* 1991a), which they suspected was due to the inhibition of host DNA

synthesis. A later study directly demonstrated that P protein was inhibiting host cell DNA replication at the stage of initiation (Datta *et al,* 2005a). Host strain mutations conferring resistance to P-killing mapped to the *dnaA* gene (Datta *et al,* 2005a) and it was demonstrated that P protein interfered with DnaA activity (Datta *et al,* 2005b), suggesting that the lethal phenotype was due to inhibition of DnaA, an important host transcription factor (Messer and Weigle, 1996) and replication initiator protein (Fuller *et al,* 1984; Bramhill and Kornberg, 1988).

1.2.4.2. The *E. coli* DnaB Replicative Helicase

DnaB is the primary replicative helicase of *E. coli.* As such, DnaB has three essential functions in cellular DNA replication. Firstly, DnaB functions as a helicase to unwind double stranded (ds) DNA; using the energy provided by ATP hydrolysis to promote the advancement of the growing replication fork (LeBowitz and McMacken, 1986). Secondly, DnaB functions as a "mobile promoter" in the general priming reaction (McMacken *et al,* 1977; Zyskind and Smith, 1977), to aid DnaG (primase) in producing RNA primers for extension by DNA polymerase III (once for leading strand synthesis and multiple times for lagging strand synthesis). Thirdly, DnaB is able to promote the progression of Holliday Junctions (branch migration), believed to be important in the repair of DNA damage occurring near advancing replication forks (Kaplan and O'Donnell, 2002; Bujalowski, 2003).

The product of the *dnaB* gene is a 470 aa monomer. Each DnaB monomer is composed of two structural domains, separated by a large, flexible linker region (Nakayama *et al,* 1984a; Nakayama *et al,* 1984b). Structure-function studies of partial

proteolysis products of DnaB suggest that the N-terminal domain of DnaB is involved in protein-protein interactions (Nakayama *et al,* 1984a; Lu *et al,* 1996). DnaB interacts with many proteins: with DnaA (Sutton *et al,* 1998), DnaC (Wickner and Hurwitz, 1975), DnaG (Lu *et al,* 1996), SSB (Biswas *et al,* 2002), the Tau subunit of DNA polymerase III (Gao and McHenry, 2001), λ P (Klein *et al,* 1980), P1 ban (Sclafini and Wechsler, 1981) and RNA polymerase (McKinney and Weschsler, 1983). The C-terminal domain is essential for DnaB's various enzymatic activities (*i.e.* for ATP binding, ATP hydrolysis, ssDNA binding, dsDNA binding and for DnaB oligomerization) (Nakayama *et al,* 1984a). The N-terminal domain is believed to be rigid in structure, while the flexible linker is important in altering the conformation of the large C-terminal domain (Nakayama *et al,* 1984a). The functional DnaB protein, a hexamer comprised of six single DnaB monomers and up to six ATP molecules (Reha-Krantz and Hurwitz, 1978; Arai *et al,* 1981), is present in limiting amounts in the cell (*i.e.* approximately 20 DnaB hexamers per cell) (Udea *et al,* 1978; Nakayama *et al,* 1984b).

The role of DnaB during *E. coli* replication initiation has been well characterized. DnaB forms a complex with DnaC ($DnaB_6$-$DnaC_6$). In this complex, DnaC acts to target DnaB to the bacterial origin of replication, *ori*C. As DnaB and DnaC are produced at similar levels, the majority of DnaB in a cell is likely bound to DnaC (Biswas and Biswas, 1987). Free DnaB has an intrinsic ssDNA binding activity; however in the DnaB-DnaC complex, DnaB's ssDNA binding activity is inhibited (Arai and Kornberg, 1981a; Kobori and Kornberg, 1982; Lanka and Schuster, 1983; Biswas and Biswas, 1987). This regulation prevents DnaB from

binding to random ssDNA and promoting promiscuous DNA unwinding. DnaC's cryptic ssDNA binding activity is only active in the DnaB-DnaC complex and DnaC specifically binds only to single stranded *ori*C DNA, ensuring that DnaB only unwinds dsDNA at an active *E. coli* replication fork (Learn *et al,* 1987). DnaC is believed to interact directly with the ssDNA caused by DnaA binding to *ori*C, while DnaB directly interacts with DnaA (Sutton *et al,* 1998) bound at *ori*C. Once DnaB is brought to the origin, it is transferred to the ssDNA in a complex series of reactions involving DnaC's ssDNA binding activity, ATP hydrolysis, several DnaB-DnaC conformational changes (Galletto *et al,* 2004b), DnaB's re-activated ssDNA binding activity and perhaps the release of DnaC from the entire complex (San Martin *et al,* 1998; Davey *et al,* 2002; Galletto *et al,* 2003; Flowers *et al,* 2003). DnaB is capable of changing its conformation several ways, as a result of binding and releasing DNA and of binding and hydrolyzing ATP (Jezewska and Bujalowski, 1996; Jezewska *et al,* 1996; Yu *et al,* 1996; Galletto *et al,* 2003; Flowers *et al,* 2003; Biswas *et al,* 2004). Each consecutive conformational change is believed to be an essential component for proper enzyme activity. Once DnaB is loaded onto the ssDNA, it begins unwinding the remaining dsDNA of the origin, allowing DNA polymerase III to synthesize DNA (Galletto *et al,* 2004a).

DnaB also plays an important role in the general priming reaction, necessary for both leading and lagging strand DNA synthesis (Arai and Kornberg, 1979). Each Okazaki fragment of the lagging strand is primed by a small RNA primer initiated by DnaG primase. DnaG directly interacts with DnaB (Lu *et al,* 1996; Thurlway *et al,* 2004), allowing DnaG to lay down each RNA primer at specified sites (McMacken *et*

al, 1977; Zyskind and Smith, 1977; Arai and Kornberg, 1981b; Arai and Kornberg, 1981c; LeBowitz and McMacken, 1986).

It has been recently demonstrated that DnaB is capable of binding to dsDNA, enabling it to drive branch migration of Holliday junctions, removing bound proteins in the process (Kaplan and O'Donnell, 2002; Bujalowski, 2003). This process is thought to be important in recombination-mediated repair of DNA lesions encountered by the moving replication fork (Kaplan and O'Donnell, 2002; Bujalowski, 2003).

Many DnaB structure-function studies have been undertaken in an attempt to understand the series of complex conformation changes essential to enzymatic function. Electron microscopy data shows that the hexamer has an asymmetric structure, with one face of the molecule having 3-fold symmetry (triangular shape) and the opposite face having 6-fold symmetry (round shape) (San Martin *et al,* 1995). The molecule appears as three outer, stain-excluding regions surrounding six massive lobules, with a channel through the molecule (San Martin *et al,* 1995; Yu *et al,* 1996). The ssDNA is passed through the interior channel (Jezewska *et al,* 1998a; Jezewska *et al,* 1998b). How the DNA enters the channel is unknown. It is believed that the six massive lobules are the large C-terminal domains of the six DnaB monomers, while the three smaller areas (seen only on one face of the hexamer) represent the small N-terminal domains, present as dimers connected to the large C-terminal lobules through the flexible hinge regions. The 3-fold symmetry seen on one face is partially explained by the finding that the hexamer is actually comprised of three DnaB dimers (San Martin *et al,* 1995).

The hexamer has three to four readily identifiable conformational states

(Jezweska and Bujalowski, 1996; Flowers *et al,* 2003), depending on the presence of DNA, ATP, ADP or protein co-factors. It is thought that global conformational changes, induced by an ATP/ADP switch (Davey *et al,* 2002), are essential for the sequential binding to and release of ssDNA required for DnaB to track processively down the DNA in a 5'to 3' direction, opening up the replication fork (Jezewska and Bujalowski, 1996).

The DnaB-DnaC complex has also been examined in an attempt to elucidate the mechanism of DnaB loading onto ssDNA. Six DnaC monomers bind to the DnaB hexamer on the face having 6-fold symmetry. The DnaB-DnaC complex has 3-fold symmetry on both faces (San Martin *et al,* 1998). There is no evidence for the existence of independent DnaC dimers (Galletto *et al,* 2003; Galletto *et al,* 2004b). However, the six DnaC monomers present in the DnaB-DnaC complex are not identical (Barcena *et al,* 2001). In fact, it appears that the DnaC monomers actually bind DnaB forming a trimer of dimers (*i.e.* three dumbell shaped DnaC "dimers" are bound to the DnaB hexamer). It is likely that the dumbbell shaped "dimers" form because DnaC monomers undergo conformational changes upon interaction with DnaB (Barcena *et al,* 2001; Galletto *et al,* 2003; Galletto *et al,* 2004b).

While an enormous amount of effort has gone into the structure-function studies, the mechanism of DnaB loading onto ssDNA remains elusive. What has been realized is the vast complexity of DnaB interactions near the origin of replication. It is known that DnaA plays some role in the loading event (Marszalek *et al,* 1996; Sutton *et al,* 1998), but the mechanism is unknown. As previously mentioned, DnaB interacts with SSB (Biswas *et al,* 2002), DnaG (Lu *et al,* 1996) and DNA polymerase III (Gao

and McHenry, 2001). It remains to be found whether any of these factors are involved in, or inhibit, loading. And finally, how seemingly unbroken ssDNA enters the inner channel of the pre-formed DnaB hexamer may remain the biggest mystery of all.

1.2.4.3. P-DnaB Complex Formation

In order to promote phage DNA synthesis, the λ P protein must recruit the host replicative helicase, DnaB, to the phage origin of replication. As previously noted, DnaB is present in very limited amounts in the cell (*i.e.* 20 hexamers), and is found in a stable complex with its loading partner, DnaC (Biswas and Biswas, 1987). In order to ensure that enough DnaB is commandeered to *ori*λ, the λ P protein has evolved to become a very strong competitor for the rare DnaB hexamer (Konieczny and Marsalek, 1995). In fact, P is able to dissociate preformed DnaC-DnaB complexes, forming P-DnaB complexes instead, which now target the helicase to an *ori*λ-O complex, thus inhibiting further *E. coli* DNA synthesis (Mallory *et al,* 1990). The ability of P to dissociate DnaB-DnaC complexes has only been demonstrated *in vitro*, and it has not been determined if this is actually possible *in vivo,* under physiological conditions. It has been reported that there are two to six P monomers bound to every DnaB hexamer (Mallory *et al,* 1990; Osipiuk *et al,* 1993; Learn *et al,* 1997). The possibility exists that a transient P-DnaB-DnaC complex could form, but there is no experimental evidence for the existence or activity of such a complex (Mallory *et al,* 1990; Learn *et al,* 1997). As in the DnaC-DnaB complex (Lanka and Schuster, 1983), DnaB present in the P-DnaB complex is enzymatically inactive (Biswas and Biswas, 1987). While DnaC's ability to hydrolyze ATP is important in re-activating DnaB's

helicase activity, P is unable to bind to or to hydrolyze ATP, leaving DnaB in a "dead-end" complex (Biswas and Biswas, 1987). Uncomplexed DnaB (free DnaB hexamer) has an intrinsic ssDNA binding activity. Upon interaction with either DnaC or P, its intrinsic ssDNA binding activity is inhibited. DnaB is always complexed with a loading partner, either DnaC or P, whose inhibitory activity prevents DnaB from binding to ssDNA and arbitrarily unwinding dsDNA anywhere in the genome (Learn *et al,* 1997). However, the P protein present in the P-DnaB complex (or the DnaC protein in the DnaC-DnaB complex) acquires an ability to interact with ssDNA, which thereupon enables the loading of DnaB at the appropriate region (*i.e.* at a replication-ready origin).

1.2.4.4. P-DnaB Complex in λ Replication Initiation

The P-DnaB complex is able to bind to ssDNA through the P subunits. However, the ssDNA binding activity of P is enhanced by the λ O protein, present in the first stage replication nucleoprotein structure at *ori*λ, *i.e.* the O-some (Learn *et al,* 1997). It has been suggested that P must be complexed to DnaB in order to efficiently bind O (Alfano and McMacken, 1989a; Dodson *et al,* 1989), although earlier studies demonstrated a complex between O and P, independent of DnaB (Zylicz *et al,* 1984; Wickner and Zahn, 1986). The increased binding affinity of P-DnaB to *ori*λ-O ensures that a functional second stage nucleoprotein replication complex, *ori*λ-O-P-DnaB, is formed. In this second stage complex, DnaB remains to be re-activated for its helicase activity and loaded onto the ssDNA at *ori*λ.

1.2.5. Activation or Firing of *ori*λ

The tight coupling between P and DnaB must be broken in order for DnaB's helicase activity to be reactivated and for the proper loading of active DnaB on to the ssDNA at *ori*λ. The activation of *ori*λ can be divided into two stages – the removal of P from DnaB and transcriptional activation.

1.2.5.1. Heat Shock Protein-Mediated Partial Disassembly of *ori*λ-O-P-DnaB

Three host encoded heat shock proteins, DnaJ, DnaK and GrpE, play an important role in λ replication initiation. Early in the 1970's, host mutations defective for λ replication were isolated (Georgopoulos and Herskowitz, 1971; Saito and Uchida, 1977). Ultimately, many of these host mutations mapped to the three heat shock genes (Yochem *et al,* 1978; Georgopoulos *et al,* 1979; Zylicz *et al,* 1985; Zylicz *et al,* 1987). The three genes, *dnaJ*, *dnaK* and *grpE*, are all positively regulated by the *rpoH* gene product, the σ^{32} factor. In turn, the heat shock proteins negatively regulate *rpoH* levels, forming an autoregulatory feedback loop (Liberek *et al,* 1995; Polissi *et al,* 1995). It has been demonstrated that λ infection induces the expression of heat shock proteins (Drahos and Hendrix, 1982), thus DnaJ, DnaK and GrpE should be present at elevated levels following λ infection (Hoffmann *et al,* 1992). These three heat shock proteins serve as molecular chaperones - preventing or dissociating protein aggregations, helping improperly or misfolded proteins to fold properly, or targeting damaged proteins for degradation (Hoffmann *et al,* 1992).

The heat shock proteins are only capable of dissociating P from DnaB if the complex between these two proteins is bound to *ori*λ-O (Osipiuk *et al,* 1993), ensuring

that P is not removed from DnaB before DnaB has reached the λ origin of replication. The first disassembly reaction involves DnaJ binding to both P and DnaB. The second step involves DnaK, which recognizes and binds to DnaJ-P. DnaK is capable of binding to P, but the presence of DnaJ enhances the P-DnaK binding affinity (Osipiuk *et al,* 1993). DnaK hydrolyzes ATP, catalyzing the release of a P monomer from the P-DnaB complex as a P-DnaK-ADP complex. During ATP hydrolysis, DnaK undergoes an important conformational change, and it is believed that the protein substrate (*i.e.* P) also undergoes a conformational change (Hoffmann *et al,* 1992). The hypothesis put forth by Hoffmann *et al.* is that in the removal of P from DnaB, DnaK (along with DnaJ and GrpE) changes the conformation of P from a native to a folded state, which is no longer able to bind to DnaB. DnaK is required at very high concentrations unless GrpE is present (Alfano and McMacken, 1989b). It is believed that GrpE enhances the initial DnaK binding to P-DnaJ and is important in the dissociation of the P-DnaK-ADP complex, allowing for the recycling of DnaK (Hoffmann *et al,* 1992; Osipiuk *et al,* 1993). There are up to six P monomers bound to every DnaB hexamer and each P monomer is likely removed from DnaB by the heat shock proteins in a step-wise manner (Learn *et al,* 1997). This leads to a step-wise release of DnaB inhibition, with each DnaB monomer being able to bind to ssDNA as each P monomer is removed (Learn *et al,* 1997). This ensures that DnaB is loaded onto DNA *in cis*, *i.e.* onto DNA immediately adjacent to the *ori*λ-O-P-DnaB complex, preventing DnaB from being loaded onto non-origin ssDNA.

Many of the π mutants of P have been shown to have a weaker binding affinity to DnaB than wild type P protein (Konieczny and Marszalek, 1995). λ phages

carrying a π mutation in *P* are usually able to replicate in *dnaJ*, *dnaK* or *grpE* defective hosts. Konieczny and Marszalek (1995) suggested that these π mutants do not need the heat shock proteins to dissociate a π-mutated P from DnaB. The weak binding affinity enables DnaB to be released and reactivated from the πP-DnaB complex spontaneously (Konieczny and Marszalek, 1995). They proposed that the π mutations convert P back to an ancestral form of P, which was a poor competitor of DnaC, resulting in poor phage DNA replication. As P evolved to become a better competitor for DnaB, its binding affinity increased to the point that an alternative mechanism was required to release DnaB from P once it was delivered to the phage origin. The lambdoid phage use the heat shock proteins, already present in high amounts due to phage infection, and functional in general protein disassociation reactions (Konieczny and Marszalek, 1995) to release DnaB from P.

Various lines of experimental evidence suggest that even when DnaB and the heat shock proteins are present at the origin, DNA synthesis does not occur (McKinney and Weschsler, 1983; Alfano and McMacken, 1988; McMacken *et al,* 1988; Mensa-Wilmot *et al,* 1988; Wegrzyn *et al,* 1996). Even though all required elements are present for the initiation of phage DNA synthesis, it is believed that replication does not begin until the origin is transcriptionally activated.

1.2.5.2. Transcriptional activation of *oriλ*

Very early in λ DNA replication studies, it was discovered that phage replication was repressed by CI protein, even when O and P proteins were present (Tomas and Bertani, 1964; Green *et al,* 1967; Dove *et al,* 1969). It was eventually

discovered that it was actually transcription from the p_R promoter (p_R transcription is regulated by CI) that was essential for replication activity. The dependence on CI regulated p_R transcription could be bypassed by phage mutations producing alternative promoters (*i.e.* c17 isolated by Packman and Sly, 1968 or ri^c mutants isolated by Dove *et al,* 1971) seemingly active when placed either upstream or downstream of the replication origin (Dove *et al,* 1969; Dove *et al,* 1971; Nijkamp *et al,* 1971; Rambach, 1973; Moore and Blattner, 1981; Furth *et al,* 1982). Transcription did not actually need to pass through the origin (Furth *et al,* 1982), leading to a hypothesis where the transcription event somehow altered the DNA topology in the origin region, allowing for DNA synthesis to initiate (Furth *et al,* 1982).

λ replication initiation requires a negatively supercoiled (underwound) DNA template (Alfano and McMacken, 1988). Lui and Wang (1987) proposed a theoretical model suggesting that transcription can change the supercoiling levels in localized DNA regions, particularly when the DNA is held immobile by bound protein complexes, such as a large nucleoprotein complex bound at *ori*λ. For every 10 bp transcribed by RNA polymerase, the DNA template must rotate once around its axis, or the RNA polymerase must rotate once around the DNA (or a combination of the two). Lui and Wang's Twin-Domain model (1987) proposed that if something prevents the transcription complex from rotating around the DNA, then the DNA must rotate. This DNA rotation would be expected to produce two domains that would eventually dissipate. The domain in front of the transcribing RNA polymerase would become over-wound (positively supercoiled) and the domain behind the transcribing RNA polymerase would become under-wound (negatively supercoiled).

Experimental *in vivo* evidence for Lui and Wang's hypothetical model indicated that, in order for persistent localized domains of supercoiling to be generated, there must be a barrier for the dissipation of the supercoiled domains (*i.e.* bound protein complexes). Topoisomerase activities (*i.e.* topoisomerase IV and DNA gyrase) play an important role in the amount of supercoiling present in a given region (Wu *et al,* 1988; Mojica and Higgins, 1996). A study by Leng and McMacken (2002) demonstrated the ability of the O-some to provide a barrier to the diffusion of transcription-induced negative supercoils. Leng and McMacken (2002) suggested that the partial unwinding of the AT rich region of *ori*λ, caused by the O-some formation, bypasses the need for transcription-mediated DNA opening of the rightward replication fork.

Another model to explain *ori*λ transcriptional activation suggests that the only effect of transcription is to remove HU (histone-like) protein from the λ DNA (McMacken *et al,* 1988; Mensa-Wilmot *et al,* 1989). Previously, it had been found that transcription (or a requirement for RNA polymerase) was required for *in vivo* λ DNA replication (Dove *et al,* 1969) or *in vitro* replication using crude cell extracts (Tsurimoto and Matsubara, 1982), but not for *in vitro* replication that was dependent upon a purified protein system (McMacken *et al,* 1988). When HU protein was added to the *in vitro* assay, RNA polymerase became a required component. It was proposed that HU protein bound to DNA surrounding *ori*λ prevented DNA replication by restraining negative supercoils, preventing open complex formation and the subsequent loading of DnaB onto ssDNA (McMacken *et al,* 1988; Mensa-Wilmot *et al,* 1989). Transcription removed HU protein, thus allowing DNA synthesis to occur

(McMacken *et al,* 1988). In summary, this model proposes that RNA polymerase has no direct role in λ replication initiation, and functions only to counteract the inhibitory effect of the HU protein (Mensa-Wilmot *et al,* 1989).

A third model for transcriptional activation of *ori*λ suggests that the host transcriptional activator DnaA and RNA polymerase are directly involved with the transfer of DnaB onto ssDNA at *ori*λ during the heat shock protein-mediated reassembly reaction (Wegrzyn *et al,* 1996). Complexes between P-DnaB and RNA polymerase (McKinney and Wechsler, 1983) and between P and DnaA (Wickner, 1978; Wegrzyn *et al,* 1996; Datta *et al,* 2005a; Datta *et al,* 2005b) have been demonstrated, suggesting a role for transcriptional activation at the mechanistic step where the heat shock proteins remove P from DnaB. It was proposed that transcription (and/or RNA polymerase directly) and DnaA are actively involved in the proper positioning of DnaB onto the SSDNA (Wegrzyn *et al,* 1996).

SeqA and DnaA are suggested to be important in the transcriptional activation of *ori*λ due to their proposed roles in the regulation of transcription from p_R (Slominksa *et al,* 2003; Wegrzyn *et al,* 1995; Glinkowska *et al,* 2003).

While the exact mechanism behind transcriptional activation remains controversial, there is little doubt that some form of transcription event(s) is essential to fire or activate the λ origin of replication. All of the models seem to suggest that the transcriptional activation event is essential for the proper loading of DnaB onto ssDNA. The loading of DnaB onto ssDNA marks the end of the initiation phase of DNA replication (Stephens and McMacken, 1997).

1.2.6. DNA Synthesis Arising From *ori*λ

λ replication from *ori*λ proceeds bidirectionally *in vivo* (Eisen *et al,* 1968; Stevens *et al,* 1971; Takahashi, 1975a; Takahashi, 1975c) in what is termed the θ mode of replication initiation. This was also shown directly by RNA-probe hybridization assays for λ replication by Hayes (1979). This process requires the loading of two active DnaB helicase hexamers onto the DNA, one to unwind the dsDNA to the left, and one to the right. Most *in vitro* assays, using purified proteins, result in unidirectional replication to the right (Anderl and Klein, 1982; Schnos *et al,* 1982; Wold *et al,* 1982; Tsurimoto and Matsubara, 1982; Dodson *et al,* 1986; Mensa-Wilmot *et al,* 1989). Several hypotheses exist to explain the blockage of the left replication fork *in vitro*. It is likely that some important cellular (or phage) factor is missing from the *in vitro* assay components. It should be noted that the previously described biochemical studies used to illustrate the various nucleoprotein structures which develop during θ replication are based on *in vitro* assays using high concentrations of purified proteins. How similar these reactions really are to the *in vivo* situation remains to be determined. No modern genetic evidence exists to support or refute the validity of these complex binding cascades.

Once DnaB is loaded onto ssDNA, it migrates processively in a 5'to 3'direction down the bound ssDNA (McMacken *et al,* 1977; LeBowitz and McMacken, 1986), using the energy from ATP hydrolysis to disrupt the hydrogen bonds between nucleotide bases in the dsDNA, causing the unwinding of the dsDNA (Galletto *et al,* 2004a). At 25°C, DnaB unwinds dsDNA at a rate of ~290 bp/s (Galletto *et al,* 2004a) in the absence of SSB protein. The ssDNA produced by DnaB

is stabilized by the binding of *E. coli* SSB (Meyer and Laine, 1990). SSB actively enhances the rate and extent of DnaB unwinding by channeling the energy of ATP hydrolysis to helicase activity (Biswas *et al,* 2002).

The actual process of θ DNA synthesis in a λ replication fork uses host encoded functions and is believed to be similar to DNA synthesis in an *E. coli* replication fork, as reviewed by Kornberg and Baker (1992). The two strands of DNA in dsDNA are complementary and antiparallel to each other, *i.e.* one strand is oriented 5' to 3' and the other 3' to 5' (Cairns, 1963). The DNA polymerase III holoenzyme is capable only of moving in a 3' to 5' direction down the leading strand, synthesizing DNA in a 5' to 3' direction. Thus, DNA polymerase III is capable only of directly synthesizing DNA on the template strand progressing from 3'to 5' (*i.e.* the leading strand) by extending a single short RNA primer synthesized by DnaG primase. The situation is more complex for the template strand progressing from 5' to 3', the lagging strand, because DNA polymerase III cannot directly synthesize DNA in the 3' to 5' direction. During lagging strand DNA synthesis, DNA polymerase III synthesizes the DNA in short segments, called Okazaki fragments, which must ultimately be joined together (Okazaki *et al,* 1968). Many RNA primers are synthesized by DnaG primase (which interacts with DnaB as described in section 1.2.4.2), approximately one primer for every two Kb of DNA, with each primer being extended by DNA polymerase III. The RNA primers are degraded and replaced with DNA by DNA polymerase I, and the resulting Okazaki fragments are joined by DNA ligase. A DnaB hexamer can directly bind to the Tau domains of two DNA polymerase III molecules (Gao and McHenry, 2001). This finding led to a model

where a DnaB hexamer, present in a replication fork, is capable of tethering the leading and lagging strand DNA polymerase III hetero-multimers (Gao and McHenry, 2001).

1.3. THE σ MODE OF λ DNA REPLICATION

1.3.1. The Switch From θ to σ Replication

After a few (five to six) rounds of θ replication from *ori*λ, λ switches to an alternate mode of DNA synthesis, the σ mode of replication (Smith and Skalka, 1966; Tomizawa and Ogawa, 1968; Takahashi, 1975c; Skalka, 1977), producing concatemers - long, linear dsDNA molecules containing tandem repeats of the λ genome (Eisen *et al,* 1968; Segawa and Tomizawa, 1971; Skalka *et al,* 1972; Takahashi, 1977). It has recently been suggested, primarily from λdv plasmid replication studies, that only a few of the approximately 50 θ DNA copies that arise from the five to six rounds of θ replication undergo σ replication (Taylor and Wegrzyn, 1995). λ switches its mode of replication approximately 15 minutes after infection (Takahashi, 1975b; Takahashi, 1977). The actual mechanism accounting for the switch and its regulation remain primarily unsolved. Concatemers were shown to develop under recombination-deficient (*int*$^-$*red*$^-$*rec*$^-$*)* conditions (Segawa and Tomizawa, 1971; Skalka, 1971), strengthening the belief that they were the natural products of the late stage of λ replication. However, the λ NinR-recombination region, containing several phage-encoded recombination genes (Kroger and Hobom, 1982) shown to be important in phage-prophage recombination events (Hayes *et al,* 2005),

was not deleted during these studies, so it has never been unequivocally proven that recombination is not involved in the production of concatemeric λ DNA.

1.3.2. σ Replication

σ replication (Gilbert and Dressler, 1968; Eisen *et al,* 1968), for which numerous complex models can be drawn, requires the appearance of a nick containing a free 3' end in one of the two strands of a double stranded circular DNA molecule. The intact strand is used as a template for DNA synthesis and the 3' end of the nicked strand is used as a primer for DNA polymerase III extension. The growing DNA strand is displaced, allowing the extension process to occur indefinitely. DNA complimentary to the displaced strand is also synthesized, in the discontinuous manner described above (*i.e.* lagging strand synthesis), to make a new double stranded linear DNA molecule (Eisen *et al,* 1968). Linear dsDNA is degraded in *E. coli* cells by the *recBCD* encoded ExoV enzyme (Enquist and Skalka, 1973). λ produces a protein called Gam, which inhibits the ExoV degradation of linear dsDNA, allowing the concatemers to remain intact in host cells (Sakaki *et al,* 1973; Enquist and Skalka, 1973).

In order to be packaged into mature phage heads, λ requires greater than unit length DNA molecules containing at least two *cos* sites (Enquist and Skalka, 1973). In the packaging process, the λ head recognizes a *cos* site on dsDNA and continues packaging DNA until a second *cos* site is reached, at which point the DNA is cleaved (Sakaki *et al,* 1973). λ efficiently packages DNA using the concatemers products produced as a result of rolling circle replication. If rolling circle replication products

are degraded (*e.g.* in a *gam*$^-$ phage), λ can package monomeric genomes using the circular dimers formed from homologous recombination (RecA or Exo-Bet dependent) between the circular monomers derived from θ replication. The developmental mechanism whereby the phage can form dimeric or multimeric DNA structures for packaging and escape the activity of ExoV has been referred to as the "Red-Rec Bypass" (Enquist and Skalka, 1973). λ needs either the products of the red (*exo* and *bet)* and *gam* genes or of the *E. coli* encoded *recA* gene to produce viable progeny. Blocking both pathways prevents the development of progeny phage (Enquist and Skalka, 1973).

1.4. UNRESOLVED ISSUES IN λ REPLICATION INITIATION

While the basic pathways involved in the initiation of bacteriophage λ have been elucidated, many important issues remain to be solved.

1.4.1. Transcriptional Activation

It has been proposed that the physical act of transcription near to a nucleoprotein complex bound at a partially denatured *ori*λ site somehow alters the DNA topology in a manner enabling the DnaB helicase to be properly positioned on the ssDNA of the replication fork. Whether this has to do with removing an inhibitory histone-like protein (HU), or with increasing negative supercoils to enhance DNA unwinding remains to be seen. Another proposal suggests that RNA polymerase plays a more direct role in activating the origin, along with the host DnaA protein, to aid in the heat shock protein mediated removal of P from DnaB.

While these models exist as a way to explain why transcription near *ori*λ is important, the actual physical mechanism remains unresolved.

1.4.2. Effect of λ *P* Expression on Host Cell Metabolism

Conflicting reports abound regarding the effect of P protein on host cells. It remains likely that host cell DNA replication initiation is halted in the presence of P protein, but the mechanism of P action and its phenotypic manifestations remain to be elucidated.

1.4.3. Initiation of Bidirectional θ Replication

In vivo, λ θ replication proceeds bidirectionally. *In vitro* DNA synthesis assays, using λdv template DNA and purified *E. coli* and phage proteins, predominately proceed unidirectionally to the right. It has been proposed that the *in vitro* assays are missing an essential component required for bidirectional initiation. It has been suggested that the host DnaA protein may be involved (Baranska *et al,* 2001). It has been demonstrated that λdv replication proceeds unidirectionally in *dnaA*$^-$ host cells (Baranska *et al,* 2001). It has also been shown that DnaA can positively regulate transcription from the p_R promoter (Wegrzyn *et al,* 1995; Szalewska-Palasz *et al,* 1998a), known to be important in transcriptional activation of *ori*λ. The model for DnaA-mediated limitation of transcriptional activation for bidirectional θ replication is as follows. As bidirectional θ replication proceeds, the number of phage genomes increases, increasing the number of p_R promoters. Eventually there is not enough DnaA to bind to and to activate the p_R promoters,

essentially titrating DnaA; and once DnaA-mediated transcriptional activation is inhibited, unidirectional *ori*λ-dependent DNA synthesis proceeds. One round of unidirectional *ori*λ-dependent DNA synthesis is thought to precede σ (*ori*λ-independent) DNA synthesis.

While this model seems straightforward, the importance of DnaA in λ replication initiation, in general, remains poorly accepted. It has been demonstrated that λ is fully capable of initiating bidirectional θ replication at 42°C in a ts DnaA host (Hayes, 1979), and λ is fully capable of replicating in *dnaA*-defective host cells (Baranska *et al,* 2001). In the 1990's, Dr. Wegrzyn's lab began publishing work proposing a role of DnaA in λdv plasmid replication. They proposed that DnaA is not required for phage replication under "normal laboratory conditions" (*i.e.* rich media and high bacterial growth rates), but in conditions closer to those found in λ's "natural environment" (*i.e.* poor media and slow bacterial growth rates), DnaA is essential for phage replication (Gabig *et al,* 1998; Wegrzyn and Wegrzyn, 2005).

1.4.4. Regulation of the Switch From θ to σ Modes of Replication

It has been suggested that λ initially replicates via the θ mode as a way of rapidly increasing genome copies, which are then used as templates for gene expression. Once enough gene copies are present, the phage switches to a mode of replication (σ) that will produce packagable DNA products (Wegrzyn and Wegrzyn, 2005). The regulation of this switch is unknown. Recent studies using λdv plasmids (Baranska *et al,* 2001) have suggested DnaA protein is important in the replication switch. They propose that bidirectional θ replication is followed by one round of

unidirectional θ replication, which is followed by σ, and that DnaA is involved in the switch from bidirectional to unidirectional θ replication, and thus the switch to σ.

Again, a role of DnaA in normal λ replication initiation remains to be demonstrated.

1.4.5. Possible Role For OOP RNA in λ Replication Initiation

Early studies involving OOP RNA demonstrated that OOP RNA expression was co-regulated with *O* and *P* expression (Hayes and Szybalski, 1973). Early results suggested that OOP RNA might play a role in λ replication initiation, helping to open up the leftward replication fork. However, in 1979, Moore *et al.* demonstrated that *oop* was not an essential component of the λ replicator. Once it was shown that OOP RNA had a role in the regulation of *cII* expression, the hypothesis that OOP was important in replication initiation was abandoned for two decades. Recent results have led to a reevaluation of the hypothesis that OOP RNA plays an important regulatory role in λ replication initiation.

1.5. GENE REGULATION VIA ANTISENSE RNA

An antisense RNA molecule can be defined as "an independent regulator of one or more target genes, whose effect is exerted post-transcriptionally" (Wagner *et al,* 2002). Using small, non-translated RNAs as regulatory molecules enables a cell to quickly and efficiently regulate gene expression. As the antisense molecule does not need to be translated into protein, it is available for activity more quickly than a protein and it is metabolically economical to produce. Many antisense molecules have

been identified which are capable of binding to and regulating several different targets. The possibility for multiple targets increases their versatility (Lease and Belfort, 2000a; Lease and Belfort, 2000b). Antisense RNAs are important for global regulation in the cell, especially in fine tuning cellular responses (Lease and Belfort, 2000a; Altuvia and Wagner, 2000). While most of the well characterized antisense RNAs negatively regulate gene expression, there are some examples where positive regulation occurs (Altuvia and Wagner, 2000). The diversity of structures, mechanisms and biological roles identified among antisense RNAs suggest that their versatility as metabolic regulators has been widely exploited by cells during evolutionary history (Altuvia and Wagner, 2000; Wagner *et al,* 2002).

1.5.1. Antisense RNA Structure and Function

Antisense RNAs are small, diffusible, untranslated RNA molecules with distinct secondary and tertiary structures (Wagner *et al,* 2002). The RNA molecule binds to target DNA or RNA at specific regions to control biological function (Simons, 1988; Wagner and Simons, 1994). The secondary structure of the antisense RNA molecule is very important for its function (Stocks and Rabbitts, 2000). Antisense RNAs consist of a series of one to three stem and loop structures (Eguchi *et al,* 1991). The loops are important for direct and specific interaction with the substrate, while the stems are important for the stabilization of the molecule (Simons, 1988; Simons and Kleckner, 1988; Wagner and Simons, 1994). The upper portions of the stems are characterized by bulges and internal loops which are essential for function (Wagner *et al,* 2002). Antisense RNAs are able to change conformations

easily to produce alternative stem-loop configurations or structures. This dynamic structure is important for the versatility of the molecules (Atluvia and Wagner, 2000). For example, the DsrA RNA molecule contains one conformation of stem-loop structures which it uses to bind to *hns* mRNA, and another (*i.e.* different) set of stem-loop structures that it uses to interact with *rpoS* mRNA. DsrA cannot bind to *hns* mRNA when it is in its *rpoS*-binding conformation, and it cannot bind to *rpoS* mRNA when it is in its *hns*-binding conformation (Lease and Belfort, 2000b). The actual binding reaction between antisense regulator and target substrate is characterized by an intricate hierarchy of intermediates, ultimately leading to an irreversible and inhibitory complex (Kolb *et al,* 2000). The multistep process is dependent on the three dimensional structure of both molecules (Stocks and Rabbitts, 2000). Preliminary binding reactions involve five to eight nucleotides in the specific loop (Wagner *et al,* 2002; Brantl, 2002a), producing a weak and reversible complex. Secondary interactions include bases in the stem structure, and also in the adjacent ss RNA region (Brantl, 2002a), producing a stronger, irreversible complex (Simons, 1988; Simons and Kleckner, 1988; Eguchi *et al,* 1991; Wagner and Simons, 1994). While the bulges and internal loops found in the stems are essential for the secondary binding reactions (Wagner *et al,* 2002; Brantl, 2002a), they are also important in the metabolic stability of the molecule, because they prevent the degradation of the molecule by double stranded RNases such as RNase III (Brantl, 2002a). The antisense RNA likely binds to the nascent RNA molecule as it is being transcribed and translated. This newly synthesized RNA molecule can be seen as a "moving target" whose secondary structure is constantly shifting, as it is interacting with RNA

polymerase and/or ribosomes (Wagner *et al,* 2002). Thus, it is difficult to predict what actual three-dimensional RNA target is bound by an antisense RNA molecule. The demonstration that artificially constructed antisense molecules are much less efficient than naturally occurring molecules suggests that the structures of these RNAs have evolved to optimize their regulatory functions (Kolb *et al,* 2000; Stocks and Rabbitts, 2000).

1.5.2. Antisense RNA Regulation of Plasmid Replication Initiation

The first antisense RNA system to be studied was the RNAI-RNAII interaction in the regulation of ColE1 plasmid replication (Tomizawa *et al,* 1981; Tomizawa, 1986). ColE1 plasmid replication initiation requires the transcription of a small primer (RNAII) for leading strand synthesis. RNAI is an antisense RNA that is complimentary to the 5' end of the RNAII primer. Binding of RNAI to RNAII causes a conformational change in RNAII, preventing an essential processing step. Thus, RNAI-RNAII interaction prevents ColE1 replication initiation. The ColE1 Rop/Rom protein enhances RNAI-RNAII binding (Tomizawa, 1990).

Antisense RNAs are important in the regulation of replication initiation from other plasmids by inhibiting the expression of plasmid encoded initiator proteins (e.g. pT181; Kumar and Novick, 1985). In all cases where antisense RNA molecules are involved in the regulation of plasmid replication initiation, the antisense RNA molecules are dependent on gene dosage; the antisense RNA regulators require strong expression and have a high turnover rate (Wagner and Simons, 1994). Constitutive gene expression and a short half life are essential, as the RNA molecules are thought

to be sensitive indicators of plasmid copy number; thus plasmid concentration changes are directly proportional to antisense RNA concentrations (Wagner *et al,* 2002; Brantl, 2002b).

1.5.3. Antisense RNA Inhibition of Translation

The most well understood mechanism of antisense RNA regulation occurs at the step of translation. The antisense RNA molecule frequently shows sequence similarity to the Shine Delgarno (SD) site on the mRNA of the gene to be regulated. Binding of the antisense RNA to the SD site occludes the ribosome from binding, preventing translation (Inouye, 1988). An example of this process involves the streptococcal plasmid, pLS1. This plasmid contains an antisense molecule, RNA II, that binds to the SD site of the *repB* mRNA, preventing ribosome binding and subsequent translation of the RepB initiator protein (reviewed in Brantl, 2002b).

Antisense RNAs can also indirectly inhibit translation by binding upstream of the SD site. The exact mechanisms of these reactions are not clear, but binding of the antisense RNA likely changes the secondary structure of the SD site, interfering with ribosome binding (Inouye, 1988). One example of this type of regulation occurs in the IncIα-IncB plasmids. Translation of the initiator genes requires the transcription of a leader sequence and the formation of a pseudoknot structure which allows a conformational change that opens up the SD site for ribosome binding. The antisense RNA binds to the SD site of the leader sequence, preventing its expression, and is also involved in preventing the psuedoknot formation; with a net result of preventing translation of the initiator gene (Nikoletti *et al,* 1988).

Antisense RNA can also activate gene expression. In the case of DsrA RNA, it binds upstream of the *rpoS* coding sequence, opening up an inhibitory stem-loop containing the SD site, enhancing mRNA stability and translation (Lease and Belfort, 2000b).

1.5.4. RNA-RNA Complexes as Targets for RNase III Degradation

Double stranded RNA complexes are rapidly degraded by RNases in the cell. The antisense RNA MicF binds to the mRNA for the *ompF* gene, forming a dsRNA-RNA complex. This complex is recognized and degraded by RNase III, leading to a decrease in *ompF* mRNA stability and translation (Aiba *et al,* 1987).

1.5.5. Other Mechanisms of Antisense RNA Activity

Traditionally, antisense RNA regulation occurs post-transcriptionally. However, there are certain instances where antisense RNA regulates the transcription of a gene. For example, pT181 antisense RNA binds to the mRNA of the plasmid pT181 *repC* initiator gene, altering the mRNA conformation so that a Rho-dependent stem-loop forms, causing premature transcription termination (Kumar and Novick, 1985). The initiation of transcription can also be inhibited by the binding of an antisense RNA to the extreme 5' end of a mRNA. For example, Tic RNA regulates *E. coli crp* gene expression in this manner (Okamoto and Freudlich, 1986).

1.5.6. Antisense RNA Molecules With Multiple Targets

As previously mentioned, regulation via antisense RNA is ubiquitous, partially

due to the metabolic cost-effectiveness of regulatory RNAs, but mostly due to the versatility of these small molecules. There exist many examples of regulatory RNAs capable of regulating multiple gene targets.

The small DsrA RNA shows sequence complimentarity to five genes – *rpoS, hns, argR, ilvlH* and *rbsD*. While the regulation of *argR, ilvlH* and *rbsD* remain hypothetical, genetic evidence exists for DsrA regulation of *hns* and *rpoS* gene expression (Lease and Belfort, 2000a). DsrA contains three possible stem-loop structures, and different stem-loops are involved in *rpoS* regulation than in *hns* regulation. DsrA positively regulates *rpoS* translation, as previously described. DsrA negatively regulates *hns* gene expression by binding to both the first and last codon of *hns* mRNA, forming a circular, partially dsRNA molecule. This molecule is not translated and is rapidly degraded (Lease and Belfort, 2000b). The *argR* and *ilvlH* mRNAs similarly contain two putative DsrA interaction sites, and it is postulated that they are regulated in an analogous manner.

The OxyS antisense RNA is responsible for the regulation of over 40 *E. coli* genes (*e.g. fhlA* and *rpoS*), using different stem-loop segments for each target sequence (Altuvia *et al,* 1998; Zhang *et al,* 1998).

1.6. OOP RNA OVERVIEW

1.6.1. Discovery of OOP RNA

In 1970, a small *l*-strand RNA was seen in the immunity-replication (*imm-rep*) region of bacteriophage λ (Syzbalski *et al,* 1970). This 4.1S RNA was observed

approximately 6 minutes after the induction of a λ prophage (Champoux, 1970), mapped to the *cII* region of the λ genome, and was proposed to play a role in DNA replication. It was suggested (Champoux, 1970) that the physical act of transcription near the origin might open up the DNA helix to expose the replication initiation site.

In 1972, Blattner and Dahlberg were examining *in vitro* transcripts arising from a λ DNA template. One transcript was a 4S *l*-strand RNA that was mapped to the *rep* region using various λ hybrid phages (*i.e.* 21, 434 and Φ80) and characterized by RNA fingerprinting. The transcript was predicted to be 81 nucleotides (nt) long and to start with a G (as opposed to the usual A). In 1973, Hayes and Szybalski described and named this small *l*-strand transcript OOP RNA (*ori*-*O*-*P* dependent). Using several cryptic prophage deletion mutants, OOP RNA was mapped to the region immediately to the left of gene *O* (Hayes and Szybalski, 1973), Fig. 1.1.

Because Rambach's *ori*λ mutations mapped immediately to the left of gene *O* (Brachet *et al,* 1970; Rambach, 1973), it was proposed that OOP RNA originated near to the origin. In the late 1970's, the mapping and sequencing of four *ori* mutations revealed that three of Rambach's replicator mutants (r93, r96 and r99) and one of Dove's replication mutants (*ti*12) fell within the coding sequence of *O* (Furth *et al,* 1977; Denniston-Thompson *et al,* 1977; Grosschedl and Hobom, 1979), and not to the immediate left of *O* as the genetic mapping data had suggested.

In 1977, Scherer *et al.* sequenced the λ *O* gene and surrounding region and *oop*'s size was determined to be 77 nt. The *oop* gene was found to be without a translational start or stop codon (Schwarz *et al,* 1978), suggesting that OOP RNA did not encode a protein. OOP RNA, as made *in vivo*, has never been sequenced to our

knowledge and so its actual length remains somewhat uncertain.

1.6.2. OOP RNA Expression

Hayes and Szybalski (Hayes, 1972; Hayes and Szybalski, 1973a; Hayes and Szybalski, 1973b; Hayes and Szybalski, 1973c; Hayes and Hayes, 1978; Hayes, 1979; Hayes and Hayes, 1979) described the regulation of p_O, the promoter for OOP RNA. *oop* transcription increased 30-100 fold within 5-10 minutes after the thermal induction of a λ prophage. The increase in transcription from p_O was co-ordinated with transcription from a region about 2000 bp downstream from *oop*, from a promoter named p_{Lit} (*i.e., l*ate *i*mmunity *t*ranscription of the distal end of *rex*). Increased transcription from both promoters required phage and host elements needed for λ DNA replication initiation (*i.e.* λ O, λ P, a functional *ori*λ, DnaB and DnaG). Surprisingly, actual DNA synthesis was not required; nalidixic acid treatment or a temperature sensitive mutation in *dnaE* encoding the alpha-subunit of DNA polymerase III, did not inhibit p_O or p_{Lit} transcription. Repressor renaturation studies showed that CI did not directly repress ongoing p_O transcription, suggesting that OOP transcription was independent of CI repression (Hayes and Hayes, 1979). CI negatively regulates transcription of p_R-*cro-cII-O-P-ren*, which is required for induction of transcription from p_O. The stimulation of transcription from p_O and p_{Lit} was gene dosage independent (Hayes, 1979), *i.e.,* not due to increased number of gene copies arising from phage replication. All the data suggested that the induction of transcription from p_O and p_{Lit} was tightly coupled to replication initiation events. Since p_O was thought to originate from the *rep* region, and its amplification was

tightly regulated with replication initiation, it was suggested that OOP RNA served as a primer for λ origin-dependent replication initiation.

1.6.3. Proposed Roles for OOP RNA in λ DNA Replication Initiation

Hayes and Szybalski tested the hypothesis that OOP RNA was a primer for DNA synthesis. *In vitro* DNA synthesis of λb2 template DNA was stimulated 7-14 fold by OOP RNA and OOP-dependent stimulation of DNA synthesis depended on DNA-free extracts from induced λ lysogens. The extracts were found to contain BLA (*b*ackground *l*owering *a*ctivity) and OSA (*O*OP *s*timulating *a*ctivity). Further characterization of these two factors remains to be done. Optimal stimulation of *in vitro* λ DNA synthesis by OOP RNA occurred at 26°C (Hayes *et al,* 1975a; Hayes *et al,* 1975b). Covalent bonding between OOP RNA and single stranded *ori*λ DNA was detected (Hayes and Szybalski, 1975). These results strengthened the hypothesis that OOP RNA was a primer for λ DNA synthesis.

In 1978, Hayes developed a model to explain the connection between p_O and *ori*λ. The "repliscriptase" model proposed that the transcription complex bound at p_O might interact with and regulate the activity of the replication initiation complex bound at *ori*λ or *vice versa*, the two complexes being separated by about 359 bp between p_O and ITN-1.

In 1979, Moore *et al.* provided data which was inconsistent with the suggestion that OOP RNA served as a primer for λ replication initiation. They argued that t_O-*oop*-p_O (the terminator, coding sequence and promoter for OOP RNA) was not an essential component of the λ replicator and was dispensable for λ DNA synthesis. The

assay system utilized was very complex, consisting of various hybrid phage constructs and helper phages growing in lysogenic cells, presumably defective for recombination. However, it is unlikely that all possible modes of recombination between the lysogenic host, test phage and helper phage were excluded. The methodologies used for strain construction and for the replication assays were not fully described, nor were these results repeated by others. For example, several of the helper phages utilized as suppliers of O and P proteins *in trans* were also capable of supplying OOP RNA *in trans*. At best, it can be said that the authors showed that *oop* is not required *in cis* for λ replication initiation. Thus, it remains unclear if the results have any bearing in a more "natural" situation, *i.e.* single phage infection or prophage induction.

In 1979, Lusky and Hobom (Lusky and Hobom, 1979a; Lusky and Hobom, 1979b) described evidence for a λ site called the "minimal" λ replicator. Again, the assay system utilized was very complex, with each cell containing multiple ColE1 derived plasmids. Plasmid retention was scored by dual antibiotic selection. Potential problems relating to incompatibility and inter-plasmid recombination were not fully resolved. For example, it has been shown that ColE1 plasmids are capable of forming long linear multimers (*i.e.* concatemers), which would be excellent templates for recombination-dependent replication (Silberstein and Cohen, 1987; Kusano *et al,* 1989), particularly if multiple plasmids are present per cell. The "mini" replicator was proposed to consist of the t_O-*oop-po* element along with a second, new component, the inceptor site (*ice*) mapping to the immediate left of t_O, and requiring the phage initiator protein, P. The model proposed that transcription initiating from p_O (or from any promoter) produced a leftward primer for DNA synthesis. The *ice* site, which has

structural similarity to transcriptional termination sites, was proposed to represent both a terminator for the replicative RNA primer, and a site that controls the "inception" of λ DNA synthesis (Lusky and Hobom, 1979a). The "maximal" λ replicator (*i.e.*, the classical *ori*λ-O-P model) was proposed (Lusky and Hobom, 1979b) to inhibit the "mini" system, which was suggested to be functional only when the maxi system was removed. Based upon this model, one would predict that *cis*-acting *ori*λ mutations could not be isolated, which appears illogical. Once again, the relevance of these results to the "natural" system remains undetermined. In 1981, Moore *et al.* demonstrated that *ice* was not essential for λ replication initiation.

Studies with λ/P22 hybrid phages (Roberts *et al,* 1976) indicated that OOP RNA and b RNA (P22 OOP analog) segregated with the immunity region rather than the replication region, implying that OOP is involved in regulation of the immunity region, and has no role in replication.

1.6.4. *oop* Expression in Repressor Establishment Transcription

The early observation (Hayes, 1972; Hayes and Szybalski, 1973a; Hayes and Szybalski, 1973b; Hayes and Szybalski, 1973c) that the short *oop* and p_{Lit} transcripts were coordinately expressed raised many questions. It was demonstrated that the p_{RM}-*cI-rex* transcript was repressed by the *cro* gene product, Fig. 1.1, shortly after prophage induction, and that *po-oop-to* and the distal p_{Lit}-*rex* transcript were turned on (Hayes and Szybalski, 1973b). The first question was whether p_O and p_{Lit} were indeed two discrete promoters, regulated by a common factor, or if the OOP (77 nt) and *LIT* (~600 nt) RNAs were the remaining stable remnants of a single longer transcript

originating from p_O. In 1976, Honigan *et al.* suggested that OOP RNA acted as a leader sequence for immunity-establishment transcription. The model suggested that OOP RNA acts as a leader for the p_{RM} promoter to transcribe *cI-rex*. The proposed immunity establishment transcript would begin at p_O, be antiterminated past t_O via the actions of the CII and CIII proteins, and continue on through genes *cI* and *rex* (Honigman *et al,* 1976). The proposed p_{RE} promoter, specific for repressor establishment transcription, was suggested to actually be p_O. Experimental results indicated the presence of a long RNA species in the region, thought to correspond to the long, antiterminated p_O transcript. However, the authors were unable to prove that this RNA was not the proposed p_{RE} transcript. The Honigman model proposed that Cro regulated the antitermination of t_O and thus the lysis-lysogeny switch. Under conditions with high Cro, p_O transcription was terminated at t_O, producing OOP RNA and allowing lytic development. Under conditions with low Cro, t_O was antiterminated to produce the long p_O-*oop*-*cI*-*rex* transcript, allowing lysogeny to predominate. This model was not, however, supported by the results of Hayes and Hayes in 1979.

Further studies indicated that while amplified repressor establishment required some of the same things as p_O-p_{Lit} transcription (*i.e.* replication requirements), repressor establishment also required the activity of the CII and CIII proteins. The regulation of the repressor establishment transcript by the Cro repressor was different than the regulation of p_O/p_{Lit} transcription by Cro (Hayes and Hayes, 1979; Hayes, 1979; Hayes, 1978). The expression of Cro from an induced λ prophage increased OOP RNA levels (*i.e.* p_O transcription) and eliminated any appearance of repressor

establishment transcription, while the opposite effect was observed from induced Cro-defective λ prophage. Thus, it was concluded that p_O-*oop*-t_O does not function as a leader sequence for repressor establishment mRNA.

1.6.5. OOP RNA as Antisense Regulator of *cII*

In 1978 it was discovered, via sequence analysis, that the last 55 nt of the *oop* gene overlap the last 55 nt in the coding sequence for the *cII* gene (Schwarz *et al,* 1978). Experimental evidence to explain the overlap did not appear until 1987. By the latter half of the 1980's, the knowledge of small, non-translated RNAs acting as antisense regulators of gene expression was becoming widespread (Takayama *et al,* 1987; Wagner *et al,* 2002). Was OOP RNA capable of regulating *cII* mRNA expression due to the 55 nt overlap in their sequences? At this time, most known antisense regulators acted by binding to the gene's Shine Delgarno (SD) sequence in the mRNA region upstream of the translational start site, interfering with ribosome binding. Regulation by binding to the 3' end of a gene was uncommon. However, one well known example of regulation at the 3' end of a transcript was *sib* site retroregulation in λ (Guarneros *et al,* 1982). After infection, the λ *int* and *xis* genes are transcribed from the p_L promoter. N-mediated antitermination results in transcription through a sequence downstream of *int*, the *sib* site. The *sib* site, when transcribed, forms a complex secondary double-stranded RNA structure recognized by RNase III, which cleaves the mRNA at the sib site, resulting in further degradation, in the 3' to 5' direction, of the entire p_L transcript, effectively regulating *int* and *xis* gene expression (Guarneros *et al,* 1982; Schmeissner *et al,* 1984).

Cells overproducing OOP RNA from a plasmid altered the plaque morphology of infecting wild type λ phage. Instead of producing turbid plaques, clear plaques were seen, which correlated to a decrease in lysogenization frequency (Takayama *et al,* 1987; Krinke and Wulff, 1987). This result produced a model in which OOP RNA served as an antisense regulator of CII protein expression, thus indirectly regulating p_{RE} transcription, which requires CII activation. The over-expression of OOP RNA should favor λ lytic development and inhibit the lysogenic mode of development. It was shown that the over-expression of OOP RNA resulted in a 100-fold decrease in the level of CII expression from a derepressed prophage in an *in vivo* assay system where CII was required for activating *galK* transcription from a cloned p_{RE} promoter (Krinke and Wulff, 1990a). In this assay the decrease in *galK* expression was dependent on RNase III activity (Krinke and Wulff, 1987; Krinke and Wulff, 1990a; Krinke and Wulff, 1990b). It was proposed that *cII* mRNA and OOP RNA formed a dsRNA complex which was recognized and cut by RNase III, leading to further degradation of *cII* mRNA by other cellular nucleases, accompanied by a corresponding drop in CII protein synthesis.

Using lysogenic cells over-expressing OOP RNA from a plasmid, it was demonstrated that RNase III cleaved the *cro-cII-O-P-ren*/OOP RNA complex. The authors referred to the transcript as *cII-O* mRNA but they produced it by inducing functional prophages. Thus, their OOP RNA target was the entire p_R-*cro-cII-O-P-ren* transcript. RNase III was shown to cleave the *cro-cII-O-P-ren*/OOP complex in the double stranded region of the *cII*-OOP overlap, 13 nt from the end of the double stranded RNA hybrid (Krinke and Wulff, 1990a; Krinke and Wulff, 1990b). The *cII*

mRNA fragment end of the p_R-*cro-cII-O-P-ren* transcript was completely degraded, while the *O* fragment end remained stable. The translation efficiency of this new truncated *O* fragment was never determined. In RNase III-defective cells, two OOP-dependent cleavages were found in the p_R-*cro-cII-O-P-ren* transcript. In contrast with the result when RNase III was active, these RNase III-independent cleavages resulted in stabilization of the *cII* fragment end and degradation of the *O* fragment end (Krinke and Wulff, 1990a). Further studies into these RNase III-independent cleavages were never undertaken.

In vivo, the CII protein has a very short half life, which in part is due to its degradation by the host FtsH protease (originally designated HflB, *h*igh *f*requency *l*ysogeny because of its known inhibition of CII activity) (Kobiler *et al,* 2004). Recent studies have implicated the terminal 16 nt of the *cII* gene as being essential for FtsH degradation, the same nucleotides capable of hybridizing to OOP RNA. Essentially, the last 17 nt of *cII* are not required for CII activity, but are necessary for CII regulation by both FtsH and OOP (Kobiler *et al,* 2002).

It was questioned if OOP RNA produced an effect on CII levels under physiological conditions. A phage with a single point mutation in p_O was stated (Krinke *et al,* 1991) to inactivate the promoter and eliminate *oop* transcription, yet this phage had the same plaque morphology, growth curve and lysogenization frequency as a p_O^+ phage. However, all of these important findings were presented as "data not shown", and thus are not accessible. The only physiological effect discussed for the p_O-defective phage was an increase in phage burst upon prophage induction (Krinke *et al,* 1991). These results disagree with previous observations by the same authors

(Krinke and Wulff, 1987), that when OOP RNA produced by an induced prophage was titrated, via the production of anti-OOP RNA, *cII* gene expression doubled; suggesting that OOP indeed played an important role in regulating CII levels. The authors ultimately proposed that OOP RNA played a minor, housekeeping role in the phage lifecycle, preventing CII protein expression late in lytic development, where it was no longer required.

1.6.6. Proposed Role for OOP RNA in Regulation of Rex Exclusion

The relevance of the p_{Lit} promoter has been pondered since its discovery in the early 1970's (Szybalski *et al,* 1970; Hayes, 1972; Hayes and Szybalski, 1973b). p_{Lit} appeared to produce an approximately 600 bp transcript corresponding to the last third of the *cI-rex* message (Hayes and Szybalski, 1973b). Much later, two rex genes, *rexA* and *rexB,* were recognized via DNA sequence analysis. It became evident that p_{Lit} might be important in the regulation of the distal *rex* gene, *rexB* (Landsmann *et al,* 1982). The presence of two p_{Lit} promoters were subsequently recognized; p_{Lit2} constitutively expresses *rexB* at low levels, while p_{Lit1} is highly expressed shortly after prophage induction, coordinately with p_O (Hayes *et al,* 1997).

The Rex Exclusion phenotype has been well documented. A λ prophage will prevent the development of an infecting T4*rII* phage, dependent on the products of the λ genes *rexA* and *rexB*, both expressed in the prophage state from the p_{RM} promoter, along with the *cI* gene. Rex Exclusion is dependent on the stoichiometric ratio of the RexB and RexA proteins in the cell (Slavcev and Hayes, 2004; reviewed in Hayes and Slavcev, in press).

How are p_O and p_{Lit1} expression connected? Are the two promoters coordinately regulated by a common factor binding to their promoters, or does OOP RNA regulate p_{Lit1} activity? Using a double lysogen system, it was demonstrated that OOP RNA, provided *in trans*, did not stimulate transcription from p_{Lit1} (Hayes, 1978), suggesting that OOP does not regulate p_{Lit1} at the level of transcription.

Sequence analysis of the p_O-*oop*-t_O and p_{Lit}-*rexB*-t_{Lit} regions showed remarkable correlations (Hayes *et al,* 1997). Both p_O and p_{Lit1} were shown to contain a nine bp conserved region with sequence similarity to the LexA binding site. This sequence was dubbed the p_O-p_{Lit} box. It appeared plausible that a common regulator, possibly LexA, could bind to these promoters at the common upstream sequence and coordinately regulate transcription initiation from them. The high degree of sequence homology in these two regions, along with their coordinate expression, suggests some sort of common regulation.

In 1997, Hayes *et al.* demonstrated that OOP RNA, over-expressed from a plasmid, suppressed the Rex Exclusion phenotype of a λ lysogen. This data strongly suggested that OOP RNA plays a direct role in regulating the Rex Exclusion phenotype. It seems likely that this may involve increasing the ratio of RexB:RexA in the cell, either by increasing RexB levels or decreasing RexA levels. Potential sites for interactions between the *cI-rexA-rexB* transcript and OOP RNA are drawn in Fig. 1.2 (Horbay *et al,* in press).

1.6.7. Revisiting the Hypothesis That OOP RNA Has a Role in λ Replication

In 1997, Obuchowski *et al.* demonstrated that the over-expression of CII could

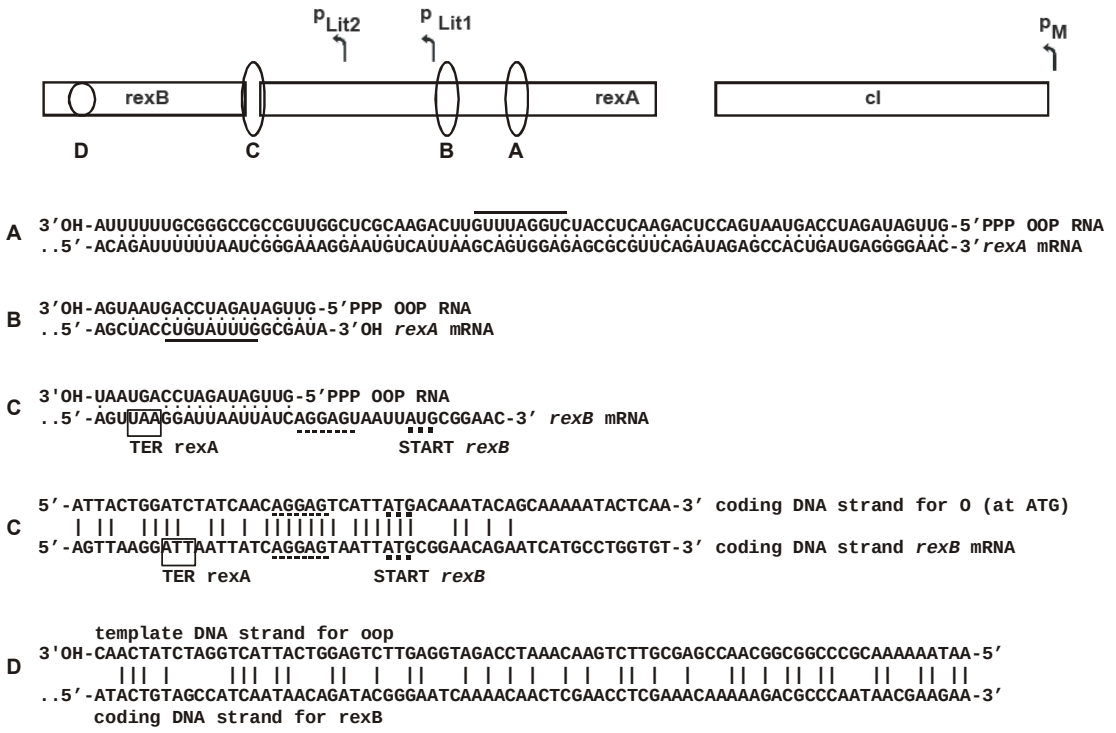

Figure 1.2. Map of λ *cI-rexA-rexB* Gene Interval, Showing Potential OOP Binding Sites. The solid line is the p_O-p_{Lit} box sequence and dashed line represents the SD sequence ahead of subsequent open reading frame. The oval shapes indicate potential OOP RNA binding sites to the *cI-rexA-rexB* mRNA. A-C show actual binding homologies (dots) within the regions indicated by the ovals. The lower C sequence indicates sequence similarity (vertical lines) between the sequence for p_O to *O* translational start and the same region for *rexB*. The circular shape (*i.e.* D) specifies a region within the *rexB* gene with sequence homology to the *oop* gene itself. Both the *rexA* and *rexB* sequences appear to contain regions with high sequence similarity to *oop*. Sequence alignments were done by SH.

inhibit λ replication initiation, which could be restored by adding excess O and P proteins *in trans*. It was proposed that transcription from p_{RE} interfered with transcription from p_R (antisense convergent transcription), required both to express the λ replication initiation genes *O* and *P* and for transcriptional activation of *ori*λ. CII over-expression was also shown to inhibit *E. coli* replication, and could be bypassed by adding excess DnaC and DnaB (Kedzierska, 2003). Because OOP RNA is responsible for inhibiting CII expression, the possibility was re-opened that OOP RNA, albeit indirectly, may have a role in regulating λ replication.

In 1998, it was noted that the copy number of a λdv plasmid was lower when the plasmid was p_O^- than when it was p_O^+ (Wrobel *et al,* 1998) and that the p_O^- mutation did not alter O protein levels. In 2002, this system was investigated in greater detail. The λdv plasmid replication and copy number was completely dependent on replication initiation from *ori*λ. It was found that a λp_O^- plasmid replicated much less efficiently during the *E. coli* relaxed response (amino acid starved, *relA*$^-$ cells), suggesting less frequent initiation from *ori*λ (Potrykus *et al,* 2002). It was proposed that transcription from p_O, not OOP RNA directly, was necessary to enhance replication from *ori*λ. As noted previously, transcription arising from p_O might change the topology of the DNA near the origin (as per Lui and Wang's Twin-Domain model), increasing the efficiency of replication initiation from *ori*λ.

Bioinformatic analyses of OOP RNA and the λ genome suggests many potential OOP binding sites. In particular, the nine bp p_O-p_{Lit} box appears to be highly conserved. For example, OOP may be capable of binding to the SD sequences of both

the *O* and *P* genes of λ, Fig. 1.3 (Horbay *et al,* in press). Subsequent binding studies may provide valuable information as to how OOP may regulate replication initiation.

1.6.8. Regulation of OOP RNA

As previously mentioned, p_O transcription requires the same signals as replication initiation, but not actual DNA synthesis. It was questioned whether there were any other elements involved in OOP regulation.

The start of the *oop* sequence and the p_O promoter contain highly conserved SOS box (LexA binding site) sequences, the only such site(s) in the entire λ genome (Sprizhitsky and Kopylov, 1983). Sprizhitsky and Kopylov proposed that *oop* was an SOS regulated gene and that induction of the SOS response removed LexA from the p_O promoter, inducing OOP expression. In 1991, Krinke *et al.* supported this hypothesis by showing that λp_O^+ has a 2-fold higher burst than λp_O^- following prophage induction by UV and that the amount of *cII* mRNA was lower in a λp_O^+ phage. They found that *lexA*$^-$ cells had 4-fold higher p_O transcription (using a p_O-*galK* fusion assay) than in *lexA*$^+$ cells. Subsequently, Lewis *et al.* directly demonstrated that *oop* was an SOS gene, regulated by LexA (Lewis *et al,* 1994). They compared LexA binding to *po* with its binding to the SOS box consensus sequence derived by Berg in 1988, *i.e.* the perfectly symmetrical 20 bp sequence TACTGTATATATATACAGTA. The LexA binding affinity at p_O was lower than for most SOS regulated genes.

In 1987 it was found that induction of the SOS response caused abnormal (*i.e.* too many) re-initiation events from *ori*λ. These abnormal initiation events were deemed to be due to supercoiling requirements for initiation becoming lax during the

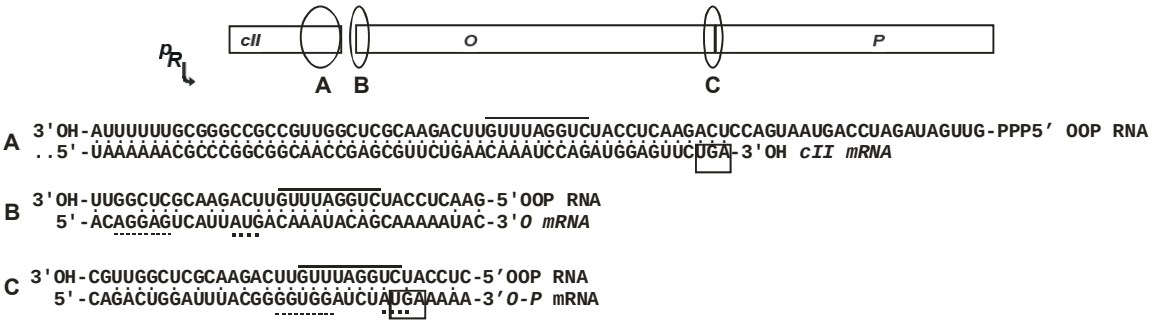

Figure 1.3. Potential OOP RNA Interaction Sites from *cII-P*. The last 55 nt of OOP RNA overlaps the last 55 nt of *cII* mRNA, resulting in a perfect 55 bp sequence homology. OOP RNA shows a high degree of sequence homology to the SD sites (dashed lines) of both the *O* and *P* genes. The sequence for C reveals the overlapping translational stop (boxed sequence) for *O* and AUG start sequence for *P*. The solid line covers the p_O-p_{Lit} box sequence within OOP RNA. While *cII*-OOP interactions have been definitively proven, all other potential OOP interactions remain hypothetical and are based upon sequence similarity. Sequence alignments done by SH.

SOS response. This abnormal re-initiation effect was shown to be LexA-dependent and RecA-independent (Schnos and Inman, 1987), which is identical to the SOS requirements for OOP induction. It may be possible that SOS induction of OOP RNA is responsible for the increased re-initiation seen at *ori*λ.

OOP RNA, as synthesized *in vivo* from an induced cryptic prophage, was found by pulse-labeling studies to have a half life of approximately 4.5 minutes (SH, unpublished). Wegrzyn's group reported that the OOP RNA half-life was shorter, about one and a half minutes (Szalewska-Palasz *et al,* 1998b). Gross and Hollatz (1988) reported that RNA transcripts terminated by the OOP terminator t_O were very labile, compared to transcripts from other terminators. The enzyme PAPI is encoded by the *E. coli* gene *pcnB*. PAPI is responsible for RNA polyadenylation, which can increase the degradation of a tagged RNA transcript. Bacteriophage λ has a very low frequency of lysogenization in $pcnB^-$ hosts, meaning that most phages are channeled into the lytic cycle, therefore, it was suggested that PAPI activity somehow stimulates lysogeny. Upon prophage induction in $pcnB^-$ cells, the observed level of CII protein was reduced compared to CII levels in $pcnB^+$ cells. The over-expression of CII and/or CIII proteins was able to suppress the loss of PAPI activity (Wrobel *et al,* 1998). It was tested whether the decrease in CII level in the $pcnB^-$ cells was caused by increased OOP RNA stability. It was found that OOP RNA was polyadenylated at its 3'OH end by PAPI and that OOP RNA was more abundant in $pcnB^-$ cells than in $pcnB^+$ cells (Wrobel *et al,* 1998). This was explained on the basis that the half life of OOP RNA was found to be three times longer in $pcnB^-$ than in $pcnB^+$ cells (*i.e.* 4.3 vs. 1.4 min) (Szalewska-Palasz *et al,* 1998b). Both the polyadenylated and non-polyadenylated

OOP RNAs were able to bind *cII* mRNA (Szalewska-Palasz *et al,* 1998b). PAPI levels increased as the bacterial growth rate decreased and OOP RNA was polyadenylated by PAPI more efficiently in slow (vs. fast) growing cells, resulting in increased CII, and enhanced lysogeny (Jasiecki and Wegrzyn, 2003).

1.6.9. The *oop* Region Is Highly Conserved Among Lambdoid Bacteriophages

In 1978 it was recognized that the phages P22, Φ80 and λ all contained "*oop*-like" genes near their respective origins (Hayes, 1978). Because these genes appeared to be evolutionarily conserved, it was proposed that they had an important role in the phage lifecycle.

The NCBI nucleotide sequence database was used to search for phages containing a p_O region similar to λ. Alignments consisting of approximately 150 bp containing the regions from the "*cII*-like" gene through the "*O*-like" gene were produced (Horbay *et al,* in press). This search revealed a highly conserved 33 bp sequence within 22 sequenced phage genomes. The 33 bp region consists of the first 13 bp of the *oop* gene, the -10 region for the *oop* promoter, the SD sequence and ATG start codon for an "*O*-like" gene, and several of the phages contained an intact p_O-p_{Lit} box. There was also a very high degree of sequence homology among the compared phages at and immediately beyond the 3' end of the OOP RNA sequence (Horbay *et al,* in press).

The high level of sequence homology in the 33 bp area overlapping the 5' end of the *oop* sequence begs the question: why is this region so strongly conserved throughout evolution in this diverse group of phages? The homologous 33 bp region

contains overlapping regulatory elements for two genes transcribed in opposite directions. In each case, the rightward ORF represents a protein implicated in replication initiation or its regulation, while the leftward promoter has the potential to produce a small nontranslated RNA which overlaps a *cII*-like gene.

The *oop* genes themselves are less conserved at the nucleotide level (Horbay *et al,* in press). The upstream regulatory region and the 5' and 3' regions show high sequence homology, with more variation in the intervening coding sequence. This suggests that the distinct *oop* genes (*i.e.* OOP RNAs) have retained regions of regulatory importance (*e.g.* self termination stem-loop) during evolution. The predicted secondary structures of OOP RNAs (Horbay *et al,* in press) show the possibility for the formation of two or three internal stem and loop structures. The effects of the OOP sequence divergences on RNA function remains unknown.

1.6.10. Physiological Role for OOP RNA in λ Development

OOP RNA is a versatile antisense RNA, with the potential for regulating multiple target sequences. Fig. 1.3 diagrams several potential regions capable of binding OOP RNA (Horbay *et al,* in press). Only one of the potential antisense interactions has been studied in any detail (*cII* regulation). The involvement of OOP RNA in the Rex Exclusion phenotype strongly supports a hypothesis where OOP RNA directly binds to *rex* mRNA, Fig. 1.2, and affects gene expression. It is tantalizing to see the high level of sequence homologies between OOP RNA and the λ initiator genes *O* and *P* when one is proposing possible models to explain a role for OOP RNA in the regulation of λ replication, Fig. 1.3. OOP may be able to bind to the

SD sites for both the *O* and *P* genes, potentially interfering with ribosome binding and subsequent translation.

The hypothesis that OOP RNA may be involved in the regulation of multiple λ genes is plausible. Many antisense regulators (e.g. DsrA and OxyS) are capable of regulating multiple targets, using completely different regulatory mechanisms for each target (Wagner *et al,* 2002). It has been shown that the high binding specificity between an antisense RNA and its target cannot be explained by simple sequence complimentarity alone. The antisense mechanism is dependent on complex interactions between intricately folded three dimensional structures of RNA (Wagner *et al,* 2002). This makes understanding antisense regulation extremely difficult. Based on sequence similarities alone, we have suggested several potential OOP target sequences, but these hypotheses remain to be tested experimentally.

In summary, we have an understanding of how OOP RNA is involved in the regulation of *cII* mRNA, but any other potential OOP functions are at this time, hypothetical. It has been demonstrated experimentally that OOP RNA is somehow involved in regulation of the Rex Exclusion phenotype. Significant OOP/*rex* sequence homologies are likely more than co-incidental. The mechanism of OOP's involvement in Rex Exclusion remains to be determined. New experimental evidence suggests a role for OOP in modulating DNA replication in λ. A plausible mechanism is not yet known, or even what OOP might be targeting. Potential OOP binding sites in the regulatory regions of the *O* and *P* genes may provide the answers.

OOP is involved in regulating CII and potentially Rex Exclusion and DNA replication, in turn influencing lytic phage development. The expression of OOP

RNA is induced in the early stages of the lytic pathway where it may function as a developmental pivot. When made in high amounts, OOP RNA functions *i*) to down-regulate CII levels (turning off the lysogenic pathway and preventing antisense convergent transcription between p_{RE} and p_R), *ii*) to potentially turn off the Rex Exclusion Phenotype (preventing self-exclusion) and *iii*) to potentially modulate phage DNA replication (indirectly influencing the lysis / lysogeny decision).

Versatile antisense RNA regulatory molecules are ubiquitous. They provide a metabolically inexpensive and rapid method of regulating multiple target sequences. While OOP's role in regulating *cII* mRNA has been well documented, other potential targets for OOP have not been considered. Further study of OOP RNA is definitely warranted. There remains much more to be learned from this small 77 nt RNA produced by bacteriophage λ.

1.7. RATIONALE FOR THE CURRENT STUDY

Bacteriophage λ has been used as an experimental paradigm for the replicon model for about 35 years. While the basic λ replication initiation scheme has been elucidated for several decades, many elements remain unclear (see section 1.4). I wished to study two unanswered issues in λ replication initiation.

I wished to learn more about what happens to a host cell when exposed to high levels of *P* gene expression. Previous studies in the Hayes laboratory (Bull, 1995) suggested that the expression of *P* from the plasmid pHB30 was not lethal to a subset of exposed cells, and that a host defect genetically mapped to *dnaB* could suppress the potentially lethal effect of P. I wished to investigate this system in greater detail by

confirming the *dnaB* allele via sequencing and by isolating plasmid mutants defective for P-killing. In order to understand the mechanism of the P-effect, I wished to search for host mutation(s) that could alter the effect of P on host cells.

I also wished to study scenarios where OOP RNA synthesis might influence λ replication initiation. I decided to use a model system previously utilized in the Hayes laboratory (Bull, 1995) in which the presence of OOP RNA on a plasmid, in conjunction with *ori*λ, was inhibitory to the development of infecting homo- and hetero-immune lambdoid phages. In order to further understand the mechanism of this inhibition, I decided to mutate the OOP and *ori*λ elements on the inhibitory plasmids. I also wished to find spontaneous phage mutants, resistant to the inhibitory phenotype, in order to learn what phase of phage development was being inhibited.

CHAPTER 2. MATERIALS AND METHODS

2.1. BACTERIAL AND BACTERIOPHAGE STRAINS AND PLASMIDS

2.1.1. *E. coli* Strains

The bacterial strains used in the P-Interference study are presented in Table 2.1; and the bacterial strains utilized in the Inhibition Phenotype study are presented in Table 2.2.

2.1.2. Bacteriophage Strains

The bacteriophage strains used in the P-Interference study are presented in Table 2.3; and the bacteriophage strains used in the Inhibition Phenotype study are presented in Table 2.4.

2.1.3. Plasmids

2.1.3.1. Previously Constructed Plasmids Utilized in the P-Interference Study

Plasmids pHB30, pHB31, pHB33 and pHB35, all constructed by Harold Bull, were derived from pCH4, a pBR322/λ hybrid (Bull, 1995); the λ fragments are presented in Figure 2.1.

Table 2.1. *E. coli* Strains Utilized in the P-Interference Study

Strain	Relevant Genotype	Hayes Laboratory Strain No; and Source
R594 =594	F$^-$ *lac*-3350 *galK*2 *galT*22 *rpsL*179 IN(*rrnD-rrnE*)1	strain B10; Bachmann, 1987
W3350A =W3350	F$^-$ *lac*-3350 *galK*2 *galT*22 IN(*rrnD-rrnE*)1	B12; Bachmann, 1987
SA500	F$^-$ *his*-87 *relA*1 *strA*181 *tsx*-83	B161; From Campbell Hayes *et al*, 2005
Y836	SA500(λ*bio*275*cI*[Ts]857Δ431)	nY836; Hayes *et al*, 2005
Y836 *dnaB*grpD55	SA500(λ*bio*275*cI*[Ts]857Δ431) *dnaB*grpD55 *malF*3089::Tn*10* Tet^R	nY1050; K. Asai, this lab; Hayes *et al,* 2005
GM2932	As AB1157 but *mutH*34 F$^-$ *thr*-1 *leu*-6 *proA*2 *his*-4 *thi*-1 *argE*3 *lacY*1 *galK*2 *ara*-14 *xyl*-5 *mtl*-1 *tsx*-33 *strA*31 *sup*-37	B150; From M.G. Marinus
594 *clpP*::kan	*clpP*$^-$ *clpX*$^-$ Kan^R	nB276; Slavcev and Hayes, 2003
594 *clpA*::kan	*clpA*$^-$ Kan^R	nB281; Slavcev and

		Hayes, 2003
594 *clpB*::kan	*clpB*$^-$ Kan^R	nB275; Slavcev and Hayes, 2003
X9368	*hflA*$^-$ Kan^R	nB272; Gottesman *et al*, 1998
594 *hflA*::kan	*hflA*$^-$ Kan^R	nB292; This work
SG22069	*ssrA*$^-$ Cam^R	Gottesman *et al*., 1998
594 *ssrA*::cat	*ssrA*$^-$ Cam^R	nB291; This work
594 *clpP*::kan Δ*lon*	*clpP*$^-$ *clpX*$^-$ *lon*$^-$ Kan^R	nB290; Slavcev and Hayes, 2003
DE407	as DE258 but is *lexA*3 (Ind$^-$) *malB*::Tn*9* Tet^R *sulA*211 *sfiA*11(*sfiA*$^-$)	B142; From Don Ennis
594 *lexA*3(Ind$^-$)	*lexA*3(Ind$^-$) UV sensitive *malB*::Tn9 Tet^R	nB293; This work
594 *dnaB*grpD55	*dnaB*grpD55 *malF*3089::Tn*10* Tet^R resistant to λ at 42°C sensitive to λ*rep*P22	nB295; A. Chu, this lab; Chu, 2005
594 *dnaA*rpl8 (single colony 4)	*galK*$^-$ *galT*$^-$ *lac*$^-$ *thi*$^-$ Str^R su$^-$ Reported as carrying mutation *dnaA*rpl8, conferring resistance to λ P-killing	nB288; From N.C. Mandal; Datta *et al,* 2005a

	Slow growing	
594 *clpP*::kan	*dnaB*grpD55	nB294; This work
*dnaB*grpD55	*malF*3089::Tn*10* Tet^R	
	$clpP^-$ $clpX^-$ Kan^R	
AB2834 $aroE^-$	*dnaB*grpD55 thi^- tsx^R	nB83; Saito and
	resistant to λ at 42°C	Uchida, 1977
	sensitive to λ*rep*P22	
W3874 *malB*5	*dnaB*grpA80 lac^- Str^R	nB81; Saito and
	resistant to λ at 42°C	Uchida, 1977
	sensitive to λ*rep*P22	
GM2932[pHB30][a]	F^-, *mutH*34	This work
	cI[ts]857, P^+, ren^+	
594[pHB30]	*cI*[ts]857, P^+, ren^+	np249; This work
594[pHB31]	*cI*[ts]857, P^-, ren^+	np250; This work
594[pHB33]	*cI*[ts]857, P^-, ren^-	np291; This work
594[pHB35]	*cI*[ts]857, P^-, ren^-	np292; This work
W3350[pHB30]	*cI*[ts]857, P^+, ren^+	p8; This work
W3350[pHB30^{nl-42}]	non-lethal at 42°C	p132; This work
isolates #1-30	phenotypically P^-	
SA500[pHB30]	*cI*[ts]857, P^+, ren^+	np295; This work
Y836[pHB30]	SA500(λ*bio*275*cI*[Ts]857Δ431)	np301; This work
	cI[ts]857, P^+, ren^+	
Y836 *dnaB*grpD55	SA500(λ*bio*275*cI*[Ts]857Δ431)	np302; This work

[pHB30]	*dnaB*grpD55	
	*malF*3089::Tn*10* Tet^R	
	cI[ts]857, P^+, ren^+	
594 *clpA*::kan [pHB30]	$clpA^-$ Kan^R	np251; This work
	cI[ts]857, P^+, ren^+	
594 *clpB*::kan [pHB30]	$clpB^-$ Kan^R	np252; This work
	cI[ts]857, P^+, ren^+	
594 *clpP*::kan [pHB30]	$clpX^-$ $clpP^-$ Kan^R	np253; This work
	cI[ts]857, P^+, ren^+	
594 *clpP*::kan Δ*lon*	$clpX^-$ $clpP^-$ Δ*lon* Kan^R	np254; This work
[pHB30]	*cI*[ts]857, P^+, ren^+	
594 *hflA*::kan [pHB30]	$hflA^-$ Kan^R	np255; This work
	cI[ts]857, P^+, ren^+	
594 *ssrA*::cat [pHB30]	$ssrA^-$ cam^R	np288; This work
	cI[ts]857, P^+, ren^+	
594 *lexA*3 [pHB30]	*lexA*3(Ind^-)	np283; This work
	malB::Tn*9* Tet^R	
	cI[ts]857, P^+, ren^+	
594 *dnaB*grpD55	*dnaB*grpD55	np297; This work
[pHB30]	*malF*3089::Tn*10* Tet^R	
	cI[ts]857, P^+, ren^+	
594 *clpP*::kan	$clpX^-$ $clpP^-$ Kan^R	np298; This work
*dnaB*grpD55 [pHB30]	*dnaB*grpD55	

	*malF*3089::Tn*10* Tet^R	
	cI[ts]857, P^+, ren^+	
Rpl8[pHB30]	Reported as carrying mutation	np300; This work
	*dnaA*rpl8, conferring resistance	
	to λ P-killing	
	Slow growing	
	cI[ts]857, P^+, ren^+	
594[pMR45]	Amp^R	np293; This work
	cro^+ cII^+ O^+ P^+ ren^+	
	cro defect = increased p_R	
	transcription	

[a] The presence of a plasmid within a cell strain is indicated by a square bracket surrounding the plasmid, following the strain designation.

Table 2.2. *E. coli* Strains Utilized in the Inhibition Phenotype Study

Strain	Relevant Genotype	Hayes laboratory strain no.; and Source
R594 = 594	F^- *lac*-3350 *galK*2 *galT*22 *rpsL*179 IN(*rrnD-rrnE*)1	strain B10; Bachmann, 1987
W3350A = W3350	F^- *lac*-3350 *galK*2 *galT*22 IN(*rrnD-rrnE*)1	B12; Bachmann, 1987
W3350 *dnaB*grpD55	*dnaB*grpD55 *malF*3089::Tn*10* Tet^R resistant to λ at 42°C sensitive to λ*rep*P22	nB15; Bull and Hayes, 1996
594 *lexA*3	*lexA*3(Ind^-) *malB*::Tn*9* Tet^R UV sensitive	nB293 ; This work
594(λ*cI*857)[a]	*imm*λ *cI*[ts]857	nY1016 ; This work
594(λ*cI*857(*18,12*)P22)	*imm*λ *cI*[ts]857 *rep*P22	nY1111 ; This work
W3350(λ*imm*434T)	*imm*434 *cI*[ts]	Y193; Hayes and Hayes, 1986
594(λSIP) – isolates 1-10	*imm*λ, Amp^R	Hayes collection # nY1115 This work
594[pBR322][b]	rop^+	np278; This work
594[pHB27]	rop^+ ice^+ OOP^+ $ori\lambda^+$	np279; This work

594[pHB27R]	Δ*rop ice*$^{+}$ OOP^{+} *ori*λ^{+}	np267; This work
594[pHB28]	*rop*$^{+}$ *ori*λ^{+}	np268; This work
594[pHB29]	*rop*$^{+}$ OOP^{+}	np269; This work
594[pHB50]	*rop*$^{+}$ Δ*ice* OOP^{+} *ori*λ^{+}	This work
594[pHB51]	*rop*$^{+}$ Δ*ice* OOP^{+} *ori*λ^{+} 60 bp deletion between p_O and *ori*λ	This work
594[pHB51kan]	*rop*$^{+}$ Δ*ice* OOP^{+} *ori*λ^{+} 1390 bp insertion between p_O and *ori*λ Kan^{R}	This work
594[pHB52]	*rop*$^{+}$ Δ*ice* OOP^{+} *ori*λ^{+} 24 bp deletion between p_O and *ori*λ	This work
594[pHB27Rp_O^-]	Δ*rop ice*$^{+}$ OOP^{+} p_O^- *ori*λ^{+} p_O promoter -10 region contains a 5 bp mismatch	np268; This work
594[pHB27R-R45OOP]	Δ*rop ice*$^{+}$ OOP^{-} *ori*λ^{+} *oop* gene bases 2-46 replaced with non-oop sequence	np287; This work
594[pHB27RΔAT]	Δ*rop ice*$^{+}$ OOP^{+} *ori*λ^{-} AT rich region of *ori*λ	np285; This work

	deleted	
594[pHB27RΔITN1-4]	Δ*rop ice*$^{+}$ OOP^{+} *ori*λ^{-}	np289; This work
	iterons 1-4 of *ori*λ deleted	
594[pHB27RΔITN3-4]	Δ*rop ice*$^{+}$ OOP^{+} *ori*λ^{-}	np290; This work
	iterons 3-4 of *ori*λ deleted	
W3350[pHB50]	*rop*$^{+}$ Δ*ice* OOP^{+} *ori*λ^{+}	p14
W3350[pHB51]	*rop*$^{+}$ Δ*ice* OOP^{+} *ori*λ^{+}	p15
	60 bp deletion between p_O	
	and *ori*λ	
W3350[pHB51kan]	*rop*$^{+}$ Δ*ice* OOP^{+} *ori*λ^{+}	p16
	1390 bp insertion between	
	p_O and *ori*λ Kan^{R}	
W3350[pHB52]	*rop*$^{+}$ Δ*ice* OOP^{+} *ori*λ^{+}	p17
	24 bp deletion between p_O	
	and *ori*λ	
594(λ*cI*857) [pHB27R]	*imm*λ *cI*[ts]857 *rep*λ	np263; This work
	Δ*rop ice*$^{+}$ OOP^{+} *ori*λ^{+}	
594(λ*cI*857) [pHB27Rp_O^-]	*imm*λ *cI*[ts]857 *rep*λ	np264; This work
	Δ*rop ice*$^{+}$ OOP^{+} p_O^- *ori*λ^{+}	
594(λ*cI*857) [pHB28]	*imm*λ *cI*[ts]857 *rep*λ	np265; This work
	rop^{+} *ori*λ^{+}	
594(λ*cI*857) [pHB29]	*imm*λ *cI*[ts]857 *rep*λ	np266; This work
	rop^{+} OOP^{+}	

594(λ*cI*857(*18,12*)P22)	*imm*λ *cI*[ts]857 *rep*P22	np259; This work
[pHB27R]	Δ*rop ice*$^{+}$ OOP^{+} *ori*λ^{+}	
594(λ*cI*857(*18,12*)P22)	*imm*λ *cI*[ts]857 *rep*P22	np260; This work
[pHB27Rp_O^-]	Δ*rop ice*$^{+}$ OOP^{+} p_O^- *ori*λ^{+}	
594(λ*cI*857(*18,12*)P22)	*imm*λ *cI*[ts]857 *rep*P22	np261; This work
[pHB28]	*rop*$^{+}$ *ori*λ^{+}	
594(λ*cI*857(*18,12*)P22)	*imm*λ *cI*[ts]857 *rep*P22	np262; This work
[pHB29]	*rop*$^{+}$ OOP^{+}	
594 *lexA*3 [pHB27R]	*lexA*3(Ind^{-})	np282; This work
	MalB::Tn*9* Tet^{R}	
	UV sensitive	
	Δ*rop ice*$^{+}$ OOP^{+} *ori*λ^{+}	

[a] The presence of a prophage within a cell strain is indicated by a regular bracket surrounding the prophage, following the strain designation.
[b] The presence of a plasmid within a cell strain is indicated by a square bracket surrounding the plasmid, following the strain designation.

Table 2.3. Bacteriophage Strains Utilized in the P-Interference Study

Bacteriophage	**Relevant Genotype**	**Hayes laboratory lysate reference no; and Source**
λ*imm*434*cI*⁻*P*am3	*imm*434 *cI*⁻ *P*am3	664; Hayes *et al*, 1998
λ*imm*434*cI*⁻*O*am205	*imm*434 *cI*⁻ *O*am205	649; Hayes *et al*, 1998

Table 2.4. Bacteriophage Strains Utilized in the Inhibition Phenotype Study

Bacteriophage	**Relevant Genotype**	**Hayes laboratory lysate reference no.; and Source**
λpapa	wt	1001; Hayes, 1979
λ*cI*857	*cI*[ts]857	1002; Hayes, 1979
λ*cI*72	*cI*⁻	999; Hayes, 1979
λ*vir*	λ*v2v1v3*	1000; Hayes, 1979
λ*cI*90 c17	*cI*⁻ c17 promoter mutation	1006; Hayes and Hayes, 1986
λse100a	escapes Nie phenotype O_{R2} pt. mut. (C to A at 37979)	1003; Hayes and Hayes, 1986
λse101b	escapes Nie phenotype O_{R2} pt. mut. (G to T at 37985)	1004; Hayes and Hayes, 1986
λse109b	escapes Nie phenotype O_{R1} pt. mut. (G to T at 38009)	1005; Hayes and Hayes, 1986
λ*cI*857(18,12)P22	*imm*λ *cI*[ts]857 *rep*P22 λhy106	998; From S. Hilliker; Hayes *et al*, 1998
λ*imm*21*cI*⁻	*imm*21 *cI*⁻	1008; Hayes *et al*, 1998
λ*imm*434*cI*⁻	*imm*434 *cI*⁻	1007; Hayes *et al*, 1998

λhy42	*imm*Φ80:*rep*λ	996; Szpirer, 1972
λ*cI*$^{+}$Δ*cII*	*cII*$^{-}$ *oop*$^{-}$	993; Oppenheim *et al*, 2004

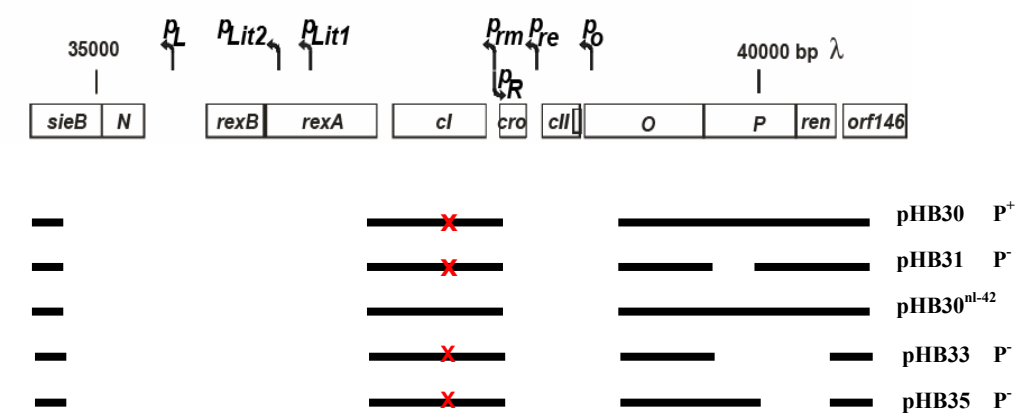

Figure 2.1. P-Interference Plasmid Maps. All plasmids are pBR322/λ hybrids. The λ DNA is represented by thick black bars. **X** represents the relative position of the 857[ts] mutation mapping 198 bp from the 5' end of the 710 bp *cI* gene. pHB30, pHB31, pHB33 and pHB35 were constructed by Harold Bull (1995). pHB30^{nl-42} was isolated during the current study (See section 3.1.4); pHB30^{nl-42} contains a point mutation within *cI* which reverts the *cI* ts allele back to wild type, preventing the expression of downstream genes at all temperatures.

pHB30 (Bull, 1995) contains the pBR322 sequences from 375-4286 and λ bases (*Bam*HI)34499-34696(*Cla*I), (*Cla*I)36965-38103(*Bgl*II) and (*Bgl*II)38814 to 40806(*Aat*II). pHB30 contains the λ genes *cI*[ts]857, *cro-O* in-frame fusion, *P* and *ren*.

pHB31 (Bull, 1995) is an in-frame *Hpa*I-*Hpa*I deletion of pHB30, removing 228 λ bases near the N-terminal end of gene *P* (*i.e.* λ bases 39608-39836).

pHB33 (Bull, 1995) is a *Hpa*I-*Stu*I deletion of pHB30, deleting 1008 bases to remove most of *P* and all of *ren* (*i.e.* λ bases 39608-40616).

pHB35 (Bull, 1995) is a *Stu*I-*Stu*I deletion of pHB30, deleting 621 bases to remove the carboxyl 285 bp of gene *P*, all of gene *ren* and most of the t_{R2} sequence (*i.e.* λ bases 39995-40616).

The plasmid construct pMR45 (Amp^R) was received from N.C. Mandal. pMR45 is a pBR322/λ construct (Maiti *et al*, 1991a and Datta *et al*, 2005a) containing λ DNA from approximately p_R through the t_{R2} terminator site (*i.e.* λ genes *cro, cII, O, P* and *ren* as well as the CI regulated p_R promoter for their transcription). A defect within Cro results in upregulated transcription from p_R (*i.e.* high constitutive levels of Cro, CII, O, P and Ren are produced).

2.1.3.2. Previously Constructed Plasmids Utilized in the Inhibition Phenotype Study

Plasmids, constructed by Harold Bull (Bull, 1995), were derived from plasmid pCH1 (Hayes *et al*, 1990) by ligating the λ34500-41731 *Bam*HI fragment into the unique *Bam*HI site of pBR322 (at 375). [Note that the λ sequences are as described by

Daniels *et al*, 1983]. The fragment orientation in pCH1 is such that the λ41731 end lies nearest the promoter for the tetracycline resistance gene of pBR322.

pHB25 (Bull, 1995) was made by digesting pCH1 with *Eco*RV to remove λ sequences 39355-41731 and pBR322 sequences 188-375. This deletes all of λ genes *P* and *ren* and the carboxyl half of gene *O*. pHB25 contains the pBR322 sequences 1-187 and 376-4361 and the λ sequence 34500-39354.

pHB26 (Bull, 1995), Fig. 2.2, was made by digesting pHB25 with *Ava*I to remove λ sequences 34500-38214 and pBR322 376-1425. This entirely deletes λ genes *sieB, N, rexB, rexA*, and *cI*. λ gene *cro* is almost entirely deleted (from the N-terminal end). λ gene *cII* remains intact, but lacks a promoter for its transcription. pHB26 retains both *po* and *ori*λ. pHB26 contains the pBR322 sequences 1-187 and 1426-4361 and λ sequences 38215-39354.

pHB27 (*rop*$^+$*to-oop-po-ori*λ or *rop*$^+$*OOP*$^+$*ori*$^+$) (Bull, 1995), Fig 2.2, was made by digesting pHB26 with *Eco*RI to remove λ sequences 39169-39354 and pBR322 sequences 4360-4361 and 1-187. The promoter for the Tet^R gene is deleted. pHB27 contains the pBR322 sequences 1426-4359 and the λ sequences 38215-39168.

pHB27R *(to-oop-p*$_O$*-ori*λ or *OOP*$^+$*ori*$^+$) (Bull, 1995), Fig 2.2, was made by digesting pHB27 with *Nde*I. This deletes the pBR322 sequences 1426-2296 and the λ sequences 38215-38358. This plasmid is deleted for the *rop Col*E1 plasmid copy number control element. pHB27R contains the pBR322 sequences 2297-4359 and the λ sequences 38359-39168.

pHB28 (*ori*$^+$) (Bull, 1995; Hayes *et al,* 1997), Fig. 2.2, was obtained by digesting pHB25 with *Bam*HI to cleave λ at 34500 and with *Bgl*II to cleave λ at 38814

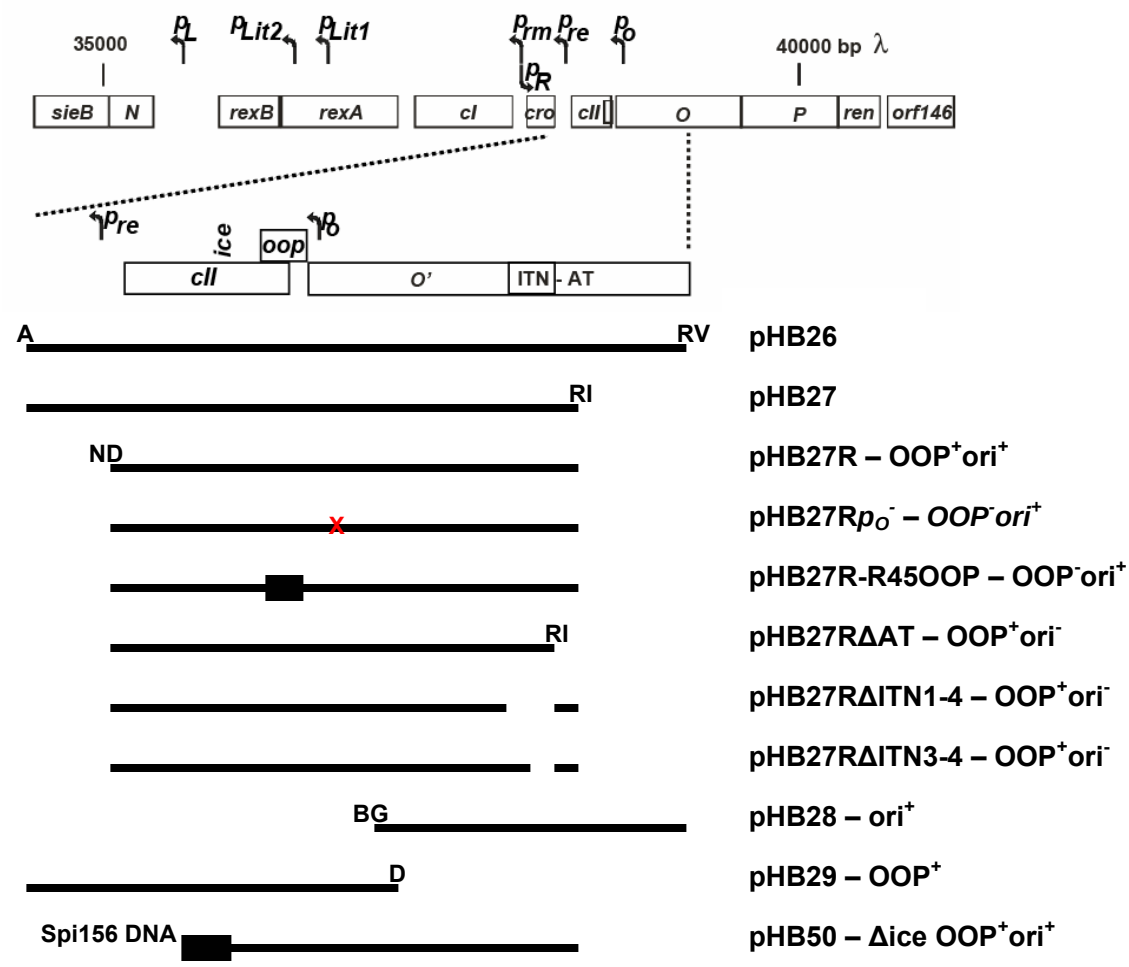

Figure 2.2. Inhibition Phenotype Plasmid Maps. All plasmids were constructed using a pBR322 backbone. Plasmid constructs containing the letter R in the name have been deleted for the plasmid copy control element Rop. pHB26, pHB27, pHB28, pHB29 and pHB50 are all *rop*$^+$. A=*Aat*II; ND=*Nde*I; BG=*Bgl*II; D=*Dra*I; RI=*Eco*RI; RV=*Eco*RV.

to remove λ sequences 34500-38814. pHB28 is deleted for *cII* and p_O, but retains the *ori*λ sequence. pHB28 contains the pBR322 sequences 1-187 and 376-4361 and the λ sequences 38815-39354.

pHB29 (OOP^+) (Bull, 1995; Hayes *et al.*, 1997), Fig. 2.2, was generated by digesting pHB26 with *Eco*RV, followed by a limited *Dra*I digest (5 minutes at 37°C). The 4791 bp fragment resulting from *Eco*RV cleavage at λ 39354 and *Dra*I cleavage at λ 38835 was identified and ligated to produce a deletion from λ 38836 – λ 39354. pHB29 is deleted for *ori*λ but retains p_O and *cII* (which lacks a promoter for its expression). pHB29 contains the pBR322 sequences 1-187 and 376-4361 and the λ sequences 38215-38835.

pHB50 (Δ*ice* OOP^+ori^+) (Bull, 1995), Figs. 2.2 and 2.3, was made by cloning the 684bp *Eco*RV-*Eco*RI fragment from λspi156Δ*nin*5 (Smith, 1975) into the *Eco*RV and *Eco*RI sites in pBR322. pHB50 contains p_O and *ori*λ, but does not contain the proposed inceptor site *ice* (Lusky and Hobom, 1979a). The 684bp fragment from λspi156Δ*nin*5 contains λ DNA from 38569-39168. The 38484-38568 fragment contains foreign (*E. coli*) DNA which has replaced the λ DNA in this region (Hayes, unpublished), thus the *ice* site has been replaced by foreign DNA. pHB50 contains the pBR322 sequence 188-4359 and the λspi156Δ*nin*5 sequence 38484-39168.

pHB51 (Bull, 1995), Fig. 2.3, was made by digesting pHB50 with *Bgl*II to remove the λ bases 38754-38814, producing a 60 bp deletion between p_O and *ori*λ. pHB51 contains λspi156Δ*nin*5 sequence 38484-38753 and 38815-39168 as well as the pBR322 sequence 188-4359.

pHB51kan (Bull, 1995), Fig. 2.3, was made by digesting pHB51 with *Bgl*II.

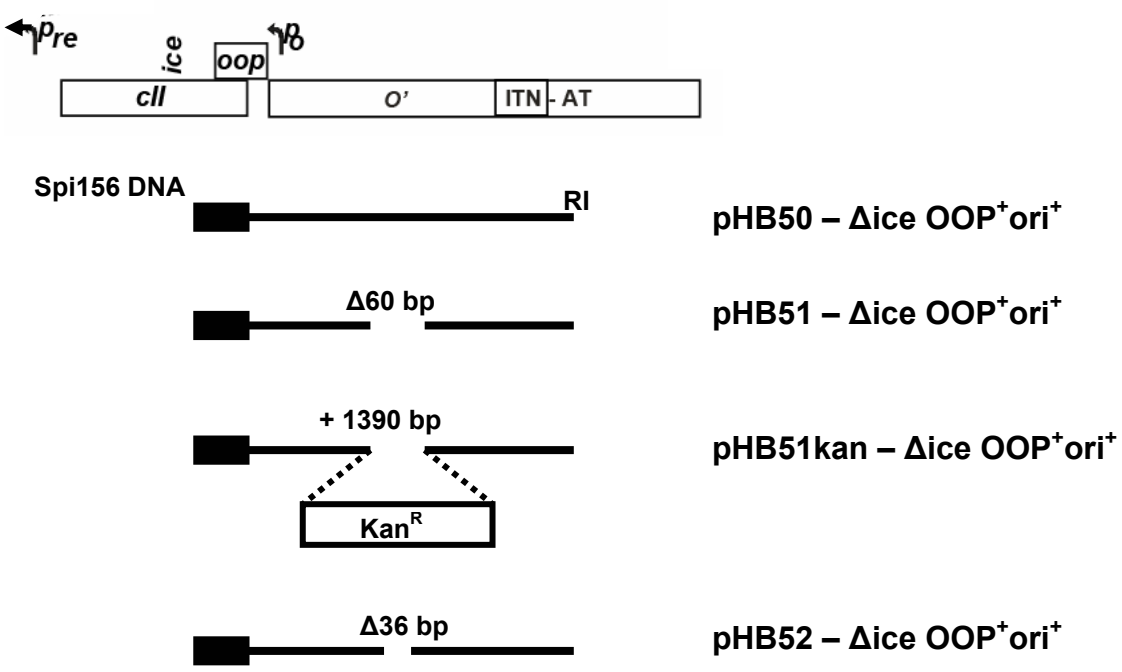

Figure 2.3. Inhibition Phenotype Plasmid Constructs Derived From pHB50. pHB51 contains a 60 bp deletion between p_O and *ori*λ. pHB51kan contains a kan^R marker cassette inserted between p_O and *ori*λ, creating a net increase of 1390 bp. pHB52 contains a partial deletion of the kan^R cassette, producing a net deletion of 36 bp between p_O and *ori*λ. All plasmid constructs were created by Harold Bull (1995).

The approximately 1450 bp *Bam*HI fragment from pUC4K (encoding the kanamycin resistance gene from Tn*903*) was then ligated into the pHB51 backbone. The promoter for the kan^R gene is positioned nearest to p_O so that its transcription is directed towards the *ori*λ sequence. pHB51kan contains λspi156Δ*nin*5 bases 38484-38753; approximately 1450 bp from Tn903; λspi156Δ*nin*5 bases 38815-39168 and pBR322 bases 188-4359. pHB51kan contains an approximately 1390 bp insert between p_O and *ori*λ.

pHB52 (Bull, 1995), Fig. 2.3, was made by partially digesting pHB51kan with *Pst*I. Fragments only digested at the *Pst*I sites flanking the kan^R gene were selected and ligated. pHB52 retains 24 bases of the Tn903 multiple cloning site adjacent to the kan^R gene, resulting in an overall deletion of 36 bp between p_O and *ori*λ. pHB52 contains λspi156Δ*nin*5 bases 38484-38753; 24 bp from Tn903; λspi156Δ*nin*5 bases 38815-39168 and pBR322 bases 188-4359.

2.1.3.2. Plasmids Constructed for Use in Inhibition Phenotype Study

All plasmid constructs were derived from pHB27R, utilizing Robert Horton's *in vitro* 'SOEing' PCR overlap extension mutagenesis technique (Horton, 1993).

pHB27Rp_O^- (*to-oop-*p_O^-*-ori*λ or *OOP*$^+$$p_O^-$*ori*$^+$). The SOEing technique was utilized to mutate the -10 region of the p_O promoter in pHB27R. Two mutated primers were designed that contained the sequence 5'GCGCG3' in place of the wt sequence 5'ATTAT3' at λ38684-λ38688, one primer containing the *l*-strand sequence λ38671-38700 (LPo3) and the other containing the *r*-strand sequence λ38700-38671 (RPo2). The pHB27R template was PCR amplified with the mutated primers and with primers

LPo1 (5' *Nde*I site and λ38357-38372) and RPo4 (5' *EcoR*I site and λ39172-39153) in a two-step PCR technique as described by Horton (1993). See Figure 2.4 for a detailed illustration of the SOEing protocol; and see Table 2.5 for actual primer DNA sequences. Essentially, the leftward portion of the template DNA (wild type pHB27R) was amplified with primers LPo1 and RPo2 (*i.e.* λ bases 38357-38700). The rightward portion of the template DNA was amplified in a second PCR with primers LPo3 and RPo4 (*i.e.* λ bases 38671-39172). Due to the complementary sequences of the mutagenic LPo2 and RPo3 primers, the two PCR products contained a 30 bp overlap between 38671-38700. The products from these two PCRs were used as the template DNA for a third PCR using primers LPo1 and RPo4. The 30 bp overlap between the DNA templates allows the templates to prime one another for Taq DNA polymerase extension, to produce 1 final PCR product spanning the entire 38357-39172 sequence, containing the desired 5 bp replacement mutation in the center of the fragment. The final PCR product was digested with *Nde*I and *Eco*RI, and ligated into the approximately 2000 bp pBR322 fragment resulting from pHB27R *Nde*I and *Eco*RI digestion. The new plasmid, pHB27Rp_O^- , Fig 2.2, was shown to be identical in sequence to pHB27R, except for the deviation at λ38684-38688 produced by the mutagenic primers.

pHB27R-R45OOP. The SOEing technique was utilized to mutate bases 2-46 of the *oop* gene coding sequence in pHB27R. Two mutated primers were designed to contain random DNA (incapable of forming secondary structure) in place of the wild type *oop* gene sequence from λ bases 38630-38674, one primer containing L-strand sequence (LROOP3) and the other containing the R-strand sequence (RROOP2). The

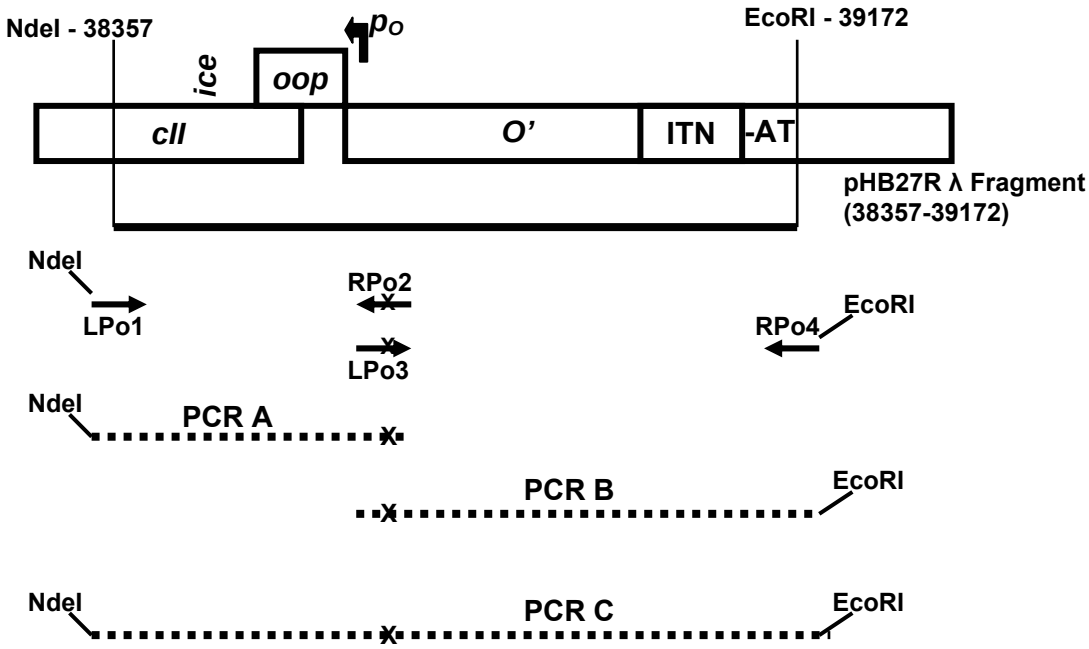

Figure 2.4. SOEing Overlap Extension Mutagenesis Technique For pHB27Rp_O^- Construction. Wild type pHB27R DNA is used as the template for PCRs A and B. PCR A was done using primers LPo1 (*Nde*I site and λ38357-38372) and RPo2 (λ38700-38671). X indicates the relative position of the 5 bp mismatch contained within primers RPo2 and LPo3 (both primers contain GCGCG at 38684-38688 in place of the wt ATTAT sequence). PCR A amplifies the λ sequence from 38357-38700, with the PCR product containing an *Nde*I site at the left end and the 5 bp mismatch at the right end. PCR B amplifies wt pHB27R DNA using primers LPo3 (λ38671-38700) and RPo4 (*EcoR*I site and λ 39172-39153). PCR B amplifies the λ sequence from 38671-39172, with the PCR product containing an *EcoR*I site at the right end and the 5 bp mismatch at the left end. PCR C uses the PCR products from PCR A and PCR B as the DNA templates, along with primers LPo1 and RPo4. PCR products A and B contain a 30 bp overlap region, which can be further extended by *Taq* DNA polymerase. The final result of PCR 3 is a λ fragment spanning the entire region, from 38357-39172, containing an *Nde*I site on the left end, an *EcoR*I site on the right end, and a 5 bp replacement mutation in the center (at 38684-38688). The PCR fragment is digested with *Nde*I and *Eco*RI and then ligated back into pBR322, creating the new construct, pHB27Rp_O^-.

pHB27R template was PCR amplified with the mutated primers and primers LPo1 and RPo4 as described by Horton (1993). The final PCR product was digested with *Nde*I and *Eco*RI, ligated into the approximately 2000 bp pBR322 fragment from pHB27R digested with *Nde*I and *Eco*RI. The new plasmid, pHB27R-R45OOP, Fig. 2.2, was found to be identical in sequence to pHB27R, except for the desired deviation at λ38630-38674.

pHB27RΔAT. The primer RΔAT-1 contains λ sequence from 39127-39113 and an *Eco*RI restriction recognition sequence at the 5' end. This primer, along with LPo1, was used to amplify the pHB27R λ DNA fragment from the left most end (*i.e.* λ38357) up to and including ITN 4 (*i.e.* λ39127). The resulting PCR fragment was digested with *Nde*I and *Eco*RI and cloned into the 2000 bp pBR322 fragment from pHB27R digested with *Nde*I and *Eco*RI. The plasmid pHB27RΔAT, Fig. 2.2, was sequenced with primers LΔPo1 and RΔAT-2 (located within pBR322) and was shown to be deleted for λ bases 39,128-39172, removing the AT rich region of *ori*λ.

pHB27RΔITN1-4. The SOEing technique was utilized to delete iterons 1-4 from pHB27R. Two hybrid primers were designed to contain sequences flanking the iterons. LΔITN1-4 contains the λ sequences 39014-39033 fused to 39120-39144, while RΔITN1-4 contains the same sequence on the R-strand (*i.e.* 39144-39120 fused to 39033-39014). These two primers, used in conjunction with LPo1 and RPo4 as described above, should result in a deletion of λ bases 39, 44-39119 (*i.e.* 87 nt of iterons 1-4). The final PCR product was digested with *Nde*I and *Eco*RI, ligated into the approximately 2000 bp pBR322 fragment from pHB27R digested with *Nde*I and *Eco*RI. The new plasmid, pHB27RΔITN1-4, Fig. 2.2, was found to be identical in

sequence to pHB27R, except for the desired deletion of bases 39044-39119.

pHB27RΔITN3-4. The SOEing technique was utilized to delete iterons 3 and 4 from pHB27R. Two hybrid primers were designed to contain sequences flanking iterons 3 and 4. LΔITN3-4 contains the λ sequences 39058-39077 fused to 39120-39144, while RΔITN3-4 contains the same sequence on the R-strand (*i.e.* 39144-39120 fused to 39077-39058). These two primers, used in conjunction with LPo1 and RPo4 as previously described, should result in a deletion of λ bases 39078-39119 (*i.e.* 41 nt of iterons 3 and 4). The final PCR product was digested with *Nde*I and *Eco*RI, ligated into the approximately 2000 bp pBR322 fragment from pHB27R digested with *Nde*I and *Eco*RI. The new plasmid, pHB27RΔITN3-4 (s.c. 2), Fig.2.2, was found to be identical in sequence to pHB27R, except for the desired deletion of bases 39078-39119.

2.2. REAGENTS, MEDIA AND GROWTH CONDITIONS

2.2.1. Solid Support Media

All experiments were carried out using TB (tryptone broth) plates (10 g Bacto-tryptone, 11 g Bacto-agar and 5 g NaCl per liter). Ampicillin was added to a final concentration of 50 μg/mL where required. Chloramphenicol was used at 20 μg/mL, tetracycline at 15 μg/mL and kanamycin at 50 μg/mL. Molten TB top agar (10 g Bacto-tryptone, 6.5 g bacto-agar, and 5 g NaCl per liter) was utilized for plating phage.

2.2.2. Culture Growth Media

All experiments were carried out using the rich growth medium TB, tryptone broth (10 g Bacto-tryptone and 5 g NaCl per liter).

Both the liquid and plate cultures were grown at 30°C, unless otherwise indicated. Liquid cultures were incubated overnight in a shaking water bath adjusted to the desired temperature. Plates were incubated inverted in a closed, temperature controlled air incubator for 16-48 hours.

2.2.3. Buffers

Φ80 buffer (1.2 g Tris pH 7.6 and 5.8 g NaCl per liter) was utilized for cell culture and phage dilutions, TE (0.01 M Tris-HCl pH 7.6-8 and 0.01 M Na_2 EDTA) and TE* (0.01 M Tris-HCl pH 7.6-8 and 0.001 M Na_2 EDTA) buffers were used for DNA storage and manipulation of DNA. TE* buffer was also used in plaque PCR protocol. TM buffer (0.01 M Tris pH 7.6 and 0.01 M $MgSO_4$) was used for phage assays (phage burst and lysate production). TBE buffer (1 M Tris pH 8, 1 M Boric acid and 0.02 M Na_2EDTA) was used to make agarose gels and as running buffer during electrophoresis.

2.3. GENERAL METHODS

2.3.1. Plasmid DNA Isolation

Plasmid DNA was isolated using Promega Wizard Plus SV Mini and Midiprep kits, as per included instructions.

2.3.2. DNA Agarose Gel Electrophoresis

0.8% agarose gels were made using TBE buffer and agarose (purchased from Sigma). Gels were run at approximately 35-60 mAmps for up to 1.5 hours. DNA was illuminated with Ethidium bromide under UV light. DNA band sizes were estimated using either a 1 Kb DNA ladder or a low DNA mass ladder, both purchased from Invitrogen. The 1 Kb DNA ladder produces DNA bands at 10 Kb, 8 Kb, 6 Kb, 5 Kb, 4 Kb, 3 Kb, 2 Kb, 1.5 Kb, 1 Kb and 0.5 Kb. The low DNA mass ladder produces DNA bands at 2000 bp, 1200 bp, 800 bp, 400 bp, 200 bp and 100 bp. Both ladders contain equimolar mixtures of each fragment, and were utilized to estimate the mass of unknown DNA samples by comparing unknown band intensities to those of the known bands.

2.3.3.Transformation of *E. coli*

The molecular procedures and the plasmid transformation of $CaCl_2$-shocked *E. coli* cells were essentially as described (Sambrook *et al*, 1989). Cells to be transformed were grown in TB to an $A_{575nm} = 0.4$, collected and cooled on ice. Cells were centrifuged at 6000 rpm for 5 minutes using a JA-20 rotor in a J2-21 Beckman centrifuge and resuspended in 0.5 X the initial volume in cold 0.01 M NaCl and recentrifuged. The pellet was resuspended in 0.5 X the initial volume of 0.03 M cold $CaCl_2$ and held on ice for 30 minutes. After centrifugation, the pellet was resuspended in 0.1 X the initial volume in cold $CaCl_2$ and held on ice for immediate use. A 10 µL aliquot of plasmid DNA was added to 0.2 mL of $CaCl_2$ cold shocked cells, the mixture held on ice for 1 hour. The mixture was heat pulsed at 42°C for 2 minutes, then

plunged into ice to chill. The mixture was then diluted into 1 mL of TB, pre-warmed to the transformation temperature, and incubated with very gentle shaking for 90 minutes. The mixture was diluted in Φ80 buffer and plated on TB+Amp plates at dilutions of 10^{-1} to 10^{-3}, and on TB plates at dilutions of 10^{-6} to 10^{-8} in order to determine efficiency of transformation. Plates were incubated at the transformation temperature for 48 hours and colony forming units (cfu) were determined. Transformation efficiencies were calculated as the cell titer (cfu/mL) on TB+Amp plates / cell titer (cfu/mL) on TB plates.

2.3.4. P1vir Transduction of *E. coli*

The molecular procedures using P1 phage were essentially as described (Miller, 1992).

2.3.4.1. Growing a P1 Lysate

The donor strain of *E. coli* was grown up overnight in TB + 0.1 % glucose. The next day, the culture was diluted and grown up in 5 mL of TB + glucose for 2 hours (to a cell titer of approximately 2.0 X 10^8 cfu/mL) and 0.25 mL of 0.1 M $CaCl_2$ was added. 0. 3mL of the mixture was removed to a clean test tube and 0.1 mL of P1 phage was added. The cell-phage mixture was incubated with shaking for 20 minutes at 37°C. A 7.5 mL volume of molten TB top agar was added to the tube and the entire mixture was poured onto 3 fresh TB plates. The plates were incubated overnight at 37°C right side up. The next morning, the top layer from the plates was scraped into a 30 mL Corex glass centrifuge tube. The plates were washed with 6 mL of TB, which

was added to the tube. A few drops of chloroform were added. The tubes were spun for 15 minutes at 10,000 rpm and the supernatant (*i.e.* the 1° P1 lysate) was collected into a sterile bottle. The entire protocol was repeated in order to produce a 2° P1 lysate for use for transduction.

2.3.4.2. P1vir Transduction

The recipient *E. coli* cells were grown up overnight in TB. For each transduction, 2 sterile 15mL Corex glass centrifuge tubes were used. Tube #1 contained 0.5 mL 15 mM $CaCl_2$ 30 mM $MgSO_4$ and 0.1-0.3 mL P1*vir*. Tube #2 (cell control) contained 0.5 mL 15 mM $CaCl_2$ 30 mM $MgSO_4$ and 0.1-0.3 mL TB. A 0.1 mL aliquot of overnight cells was added to each tube and incubated with shaking at 30°C for 20 minutes. The tubes were centrifuged at 10,000 rpm for 10 minutes. Pellets were resuspended in 0.5 mL TB+20 mM Sodium Citrate. The mixtures were then shaken at 30°C for 60 minutes and then spread on appropriate drug plates containing 20 mM Na-Citrate (to inhibit attachment of free P1 phage). A P1 control was also plated to ensure that the lysate prep is free of contaminating donor cells. Plates were incubated for 48 hours at 30°C and drug resistant colonies were selected for subsequent genetic testing.

2.3.5. PCR Primer Design

The primers used in the P-Interference and Inhibition Phenotype studies are presented in Table 2.5.

E. coli primers were designed to amplify and sequence the *dnaA* and *dnaB*

Table 2.5. Primers Utilized in P-Interference and Inhibition Phenotype Studies

Primer Name	Species	Primer Orientation	Map Position (bp)	5' to 3' Primer DNA Sequence
DnaA-1	*E. coli*	L	3880273-3880290	ACG ACC ACC TAA CGG ACC
DnaA-2	*E. coli*	R	3880756-3880739	GTA CGT GAG CTG GAA GGG
DnaA-3	*E. coli*	L	3880739-3880756	CCC TTC CAG CTC ACG TAC
DnaA-4	*E. coli*	R	3881280-3881264	CGG ATA ACC CTG GCG GT
DnaA-5	*E. coli*	L	3881264-3881280	ACC GCC AGG GTT ATC CG
DnaA-6	*E. coli*	R	3881834-3881817	GCA GGG TCT TTT CGA CGT
DnaB-1	*E. coli*	L	4262244-4262262	CGT GTT GCC ATG TGT CCT
DnaB-2	*E. coli*	R	4262943-4262926	CAT CGT GTG GCT GCT GAA
DnaB-3	*E. coli*	L	4262789-4262806	GCG TAC CAG CGA AGA TCT
DnaB-4	*E. coli*	R	4263358-4263341	ATG ATA AGC CCG ATG CCG
DnaB-5	*E. coli*	L	4263227-4263244	TTC CGG CAC CAT GGG TAT

DnaB-6	*E. coli*	R	4263813-4263796	CGC TTG CAT TTG TGT TTC
LCI-1	λ	L	37189-37206	TTG TTA TCA GCT ATG CGC
LMH29	λ	L	37905-37922	CTG CTC TTG TGT TAA TGG
RCI-1	λ	R	37996-37979	AAT AGT CAA CAC GCA CGG
RPo6	λ	R	pBR322 L 1423-1425	ATC TCG GGA AGG GCT TTA CC
			+ λ38218-38202	
LPo1	λ	L	pBR322 R 2308-2300	CAC ACC GCA TAT GGT TCG TGC AAA C
			+ λ38357-38372	
L22	λ	L	38517-38534	TGC TGC TTG CTG TTC TTG
LPG30	λ	L	38530-38547	TTG GAA CTG AGA AGA CAG
RPG6	λ	R	38569-38552	CAA TCG AGC CAT GTC GTC
RROOP2	λ	R	45nt random DNA +	CTT CTG TAC TAT TAT CTC TCT CCC TTT GCT TTT
			38629-38612	ATA CTC TCA TTA AGA ACG CTC GGT TGC CGC
LPG5	λ	L	38668-38685	CTA TCA ACA GGA GTC ATT
RPG2	λ	R	38685-38668	AAT GAC TCC TGT TGA TAG

LPo3	λ	L	38671-38700	TCA ACA GGA GTC CGC GCG ACA AAT ACA GCA
RPo2	λ	R	38700-38671	TGC TGT ATT TGT CGC GCG GAC TCC TGT TGA
LROOP3	λ	L	45nt random DNA +	TAA TGA GAG TAT AAA AGC AAA GGG AGA GAG ATA
			38675-38692	ATA GTA CAG AAG CAG GAG TCA TTA TGA CAA
LPG1	λ	L	38784-38801	AAA TAT GCT GCT TGA GGC
RPG31	λ	R	38881-38864	GTT TAT TCC ACC CAT AGG
R11	λ	R	38913-38897	GCT AAG TTG AGA ATC GG
LΔITN1-4	λ	L	39014-39033 and	AAA ACA TCT CAG AAT GGT GCC ACA AAA GAC ACT
			39120-39144	ATT ACA AAA GAA
LΔITN3-4	λ	L	39058-39077 and	CCT AAA ACG AGG GAT AAA ACC ACA AAA GAC ACT
			39120-39144	ATT ACA AAA GAA
RΔITN1-4	λ	R	39144-39120 and	TTC TTT TGT AAT AGT GTC TTT TGT GGC ACC ATT
			39033-39014	CTG AGA TGT TTT
RΔAT-1	λ	R	pBR322 L 4358-4360	AAG AAT TCC TTT TGT GTC CCC CT

			+ λ39127-39113	
RΔITN3-4	λ	R	39144-39120 and	TTC TTT TGT AAT AGT GTC TTT TGT GGT TTT ATC
			39077-39058	CCT CGT TTT AGG
RPo4	λ	R	pBR322 L 4358-4360	AAG AAT TCT CTG ACG AAT AAT CT
			+ λ39172-39153	
R9+1	λ	R	39191-39175	TGG TCA GAG GAT TCG CC
L20	λ	L	39465-39484	ACT CCG CGA TAA GTG GAC CC
RMH25	λ	R	39626-39609	CTG CTC ACG GTC AAA GTT
RMH33	λ	R	40315-40295	GCG ACG TCC CCA GGT AAT GAA TAA TTG C
R19	λ	R	40588-40571	GCT CAG TAA TGT AGA TGG
LMH32	λ	L	39531-39550	CAC AGA TCT ATA GCA AAC CAA AAC TCG ACC TGA
LΦ80-CII	Φ80	L	NCBI # X13065 [b]	GGT TAT ATG AGA GGC AC
			4139-4155	
LΦ21-CII	Φ21	L	NCBI #AJ237660	GGT CTT GTG TGC TTT CGG
			38513-38530	

RP22-18	P22	R	NCBI # NC002371	AGA ACC TTC ATC GCA TCC
			33000-32983	
LPo5	pBR322	R	NCBI # J01749	CTA TTC GAA ATT ACG CCA TCA
			26-46	
RΔAT-2	pBR322	L	NCBI # J01749	CCC CGA AAA GTG CCA CCT GA
			4266-4285	

[a] Primer orientation is designated as either "L" or "R", indicating which λ DNA strand (*l* or *r*) will be produced upon amplification. Similarly, the *E. coli* primers are designated in the same manner (even though *E. coli* DNA strands are not designated as *l* or *r*).
[b] NCBI # refers to the NCBI Genbank accession number of the DNA sequence used to construct the primer.

genes of several host strains. The *E. coli* K-12 sequence was obtained from the NCBI genome database (U00096). DnaA-1 (3880273-3880290) and DnaA-6 (3881834-3881817) were used to amplify the entire *dnaA* gene (from approximately 3880273-3881834). Primers DnaA-3 (3880739-3880756) and DnaA-4 (3881280-3881264) were sent to PBI as primers for sequencing both strands of *dnaA* from approximately 3880739-3881280. Primers DnaB-1 (4262244-4262262) and DnaB-6 (4263813-4263796) were used to amplify the entire *dnaB* gene (from approximately 4262244-4263813). Overlapping primer pairs DnaB-1 and DnaB-2; DnaB-3 and DnaB-4; and DnaB-5 and DnaB-6 were sent to NRC/PBI to sequence both strands of the entire *dnaB* gene.

Primers were designed for amplifying various regions of pHB30 and pHB30^{nl-42} isolates. See Table 2.5 for actual primer DNA sequences. The λ DNA sequence used for generating primers was that from Daniels, *et al*. (1983) as well as from the NCBI genome data base (NC001416). The λ *P* gene of the plasmid isolates was amplified using the primers L20 and R19. The primer pair L20 (λ39465-39484) and R19 (λ40588-40571) is expected to amplify a fragment 1123 nt long. The primers LMH29 (λ37905-37922) and RMH25 (λ39626-39604) were designed to amplify the plasmids from upstream of p_R to *P*, in order to search for possible insertions limiting the downstream expression of gene *P*. The expected PCR fragment, without insertions, is predicted to be 1010 or 1070 nt long (the discrepancy existed because the exact composition of pHB30 was unknown, and the *cro-O* fusion gene could be produced from one of two possible restriction sites 60 bp apart). [Note: While I will provide the sequencing results elsewhere, it is important to mention here that I

identified the *Bgl*II site at 38814 as the site used to produce the *cro-O* fusion in pHB30 construction]. The primers LCI-1 (λ37189-37206) and R20 (λ40764-40747) were used to amplify the entire λ fragment within the pBR322 backbone of pHB30, to search for possible insertions. The expected PCR fragment is 2864 nt. The primers LMH29 (λ37905-37922) and R11 (λ38913-38897) were utilized to amplify the p_R region of the plasmids. The expected PCR fragment is 297 nt. The primers LCI-1 (λ37189-37206) and RCI-1 (λ37996-37979) were designed to amplify the *cI* gene of the plasmids, with an expected PCR product of 807 nt.

The primers used for IP plasmid construction and sequencing were previously discussed in section 2.1.3.2.

The various phages utilized in the IP assay were sequenced from *oop* through *ori*λ, *i.e.* from approximately λ bases 38534-39175, to search for DNA sequence variations. This included an analysis of phages λwt, λ*cI*72, λ*cI*857, λ*cI*90c17, λvir, λse100a, λse101b, λse109b, λ*imm*434*cI*ˉ and all 10 SIP mutants. These phages were amplified with primers L22 (38517-38534) and R9+1 (39191-39175) and sent to NRC/PBI for sequencing from approximately 38534-39175 (*i.e.* from the inceptor site *ice* through *ori*λ). The corresponding region was also sequenced for phages λ*imm*21*cI*ˉ using primers LΦ21-CII and R9+1; λhy42 involved primers λΦ80-CII and R9+1, and λ*cI*857(*18,12*)P22 was amplified with primers L22 and RP22-18.

λ*cI*857 and the SIP phages 1-4 were also sequenced with primers LMH29 (37905-37922) and RPG6 (38569-38552), which amplify the o_R/p_R region, the *cro* gene and the N-terminal portion of *cII*.

2.3.6. PCR Protocols

2.3.6.1. General PCR Protocol

The general PCR mixture and protocol were as suggested by the Invitrogen insert contained with the *Taq* DNA polymerase. A 69.5 µL aliquot of sterile distilled H_2O (*i.e.* dH_2O) was mixed with 10 µL 10 X PCR buffer, 3 µL 50 mM $MgCl_2$, 2 µL 10 mM dNTPs, 10 µL template DNA, 2.5 µL L primer at 0.2 µg/mL, 2.5 µL R primer at 0.2 µg/mL and 0.5 µL *Taq* DNA polymerase at 2 Units/µL) in a thin-walled 0.5 mL microfuge tube. The PCR mixture was pulsed in a table top centrifuge and inserted into the PCR machine. The PCR machine utilized was the Thermo Hybaid PCR Sprint Temperature Cycling System. As the machine contains a heated lid, the PCR tubes can be placed directly into the block, without the addition of oil to the samples. The PCR protocol called for 3 minutes at 94°C, followed by 30 cycles of 94°C for 45 seconds, 55°C for 30 seconds, and 72°C for 1 minute per 1 Kb of DNA extended by *Taq* DNA polymerase. And finally, 10 minutes at 72°C. The PCR results were examined and quantitated by gel electrophoresis.

2.3.6.2. Whole Cell PCR Protocol

For whole cell PCR, all components remained the same as above, except the template DNA and dH_2O. Instead of 10 µL template DNA, a toothpick was stabbed into a well isolated colony and the cells were swirled into the PCR tube. In order to compensate for the decrease in total volume, the amount of dH_2O was increased from 69.5 µL to 79.5 µL. Before the addition of the *Taq* DNA polymerase, the tubes were

incubated at 95°C for 10 minutes, causing cell lysis, and then cooled at room temperature for 2 minutes. After addition of the *Taq*, the general PCR protocol was resumed.

2.3.6.3. Plaque PCR Protocol

The phage to be amplified was strip streaked onto 594 host cells overlaid onto a TB plate using sterile paper strips and incubated overnight at 37°C. The next day, 4 plaques were picked, using a sterile Pasteur pipette, into 30 μL of TE* buffer and incubated for 2 hours at room temperature to allow the release of phage particles from the agar plug. The plaque mixture was heated at 95°C for 5 minutes to melt the agar plug and to break the phage particles open, and then centrifuged in a Tabletop microcentrifuge for 5 minutes. 10 μL of the supernatant was added to the PCR tube as template DNA and the general PCR protocol was then resumed.

2.3.7. Gel Extraction and DNA Purification

PCR fragments to be sequenced were first run on a 0.8 % agarose gel to visualize DNA bands. Single DNA bands were purified and concentrated using a Qiagen PCR cleanup kit, as per instructions. In situations where multiple DNA bands were present, the band of interest was cut out of the gel and purified using a Qiagen Gel Extractor kit, as per instructions. The Qiagen PCR cleanup kit was subsequently used to further concentrate the extracted DNA. The final concentration of purified DNA samples was quantitated by running 4 μL of the sample alongside 4 μL of the low DNA mass ladder from Invitrogen and comparing the unknown band intensities

with bands of known concentration in the mass ladder.

2.3.8. DNA Sequencing

Samples to be sequenced were diluted to 20 µg/µL (unless otherwise indicated) and sent to Barry Panchuk at the National Research Council /Plant Biotechnology Institute (NRC/PBI) along with the required primers (at 4 pmol/µL). Each sample sent to PBI was sequenced on both *l* and *r* strands. Raw DNA sequences were analyzed using NCBI's BLAST program (http://www.ncbi.nlm.gov/blast/bl2seq) and via the Multialign website (http://prodes.toulouse.inra.fr/multalin/multalin .html). The sequence data was visually compared to wild type sequence and any sequence deviations were noted. The noted mutation was required to be present in both *l* and *r* strand sequences in order to be identified as a true base change.

2.3.9. Restriction Digestion Analysis

All restriction enzymes were purchased from New England Biolabs (NEB) and used as directed.

2.3.10. T4 DNA Ligase Protocol

T4 DNA ligase was purchased from New England Biolabs and used as directed. Essentially, 8 µL of each DNA fragment to be ligated was mixed with 2 µL dH_20, 2 µL T4 DNA ligase buffer and 0.5 µL of T4 DNA ligase. The mixture was pulsed in a Tabletop centrifuge and incubated at 16°C for 2 hours. The reaction was stopped by heat inactivation at 65°C for 10 minutes.

2.3.11. UV Sensitivity Assay

Cells to be tested for UV sensitivity were streaked in a line across a TB plate. Tinfoil was laid across half of the opened plate, which was then exposed to UV light (254 nm) for 20 seconds, at a distance of 30 cm from the UV light source. After UV exposure, the plates were incubated at 30°C overnight. Cells able to grow under the tinfoil covered region of the plate, but unable to grow on the UV exposed portion of the plate were deemed to be UV sensitive. Wild type and UV sensitive control cells were also exposed to UV light in the same manner and used as a comparison to the tested cells.

2.3.12. Phage Lysate Preparation Techniques

2.3.12.1. Liquid Culture Technique

Stationary phase host cells (0.3 mL) were mixed with 3 mL of molten TB top agar and poured onto a TB plate. Once solidified, approximately 0.2-0.5 mL of the phage to be used was spotted near an edge of the plate and sterile paper strips were used to dilute the phage lysate. The plate was incubated overnight at 37°C. The next morning a single plaque was picked, using a sterile Pasteur pipette, to 0.5 mL of TM buffer. The phage solution was finger vortexed and held at 37°C for 5 minutes. A mixture comprising 0.3 mL of stationary phase host cells and 0.3 mL of 0.01 M $MgCl_2/CaCl_2$ was combined with 0.3 mL of the phage suspension. The mixture was finger vortexed and then held at 37°C for 15 minutes to allow phage and cell attachment. The entire mixture was pipetted into 10-20 mL of prewarmed TB and

incubated at 37°C with vigorous shaking for 5-7 hours (or until visible cell lysis was apparent). Chloroform was added to the mixture and shaking was continued for 30 minutes. The mixture was then centrifuged at 10,000 rpm for 10 minutes and the supernatant was collected and stored at 4°C.

2.3.12.2. Plate Lysate Technique

Stationary phase host cells (0.3 mL) were mixed with 3 mL of molten TB top agar and poured onto a TB plate. Once solidified, approximately 0.2-0.5 mL of the phage to be used was spotted near the edge of the plate and sterile paper strips were used to dilute the phage lysate. The plate was incubated overnight at 37°C. The next morning a single plaque was picked, using a sterile Pasteur pipette, into 0.5 mL TM buffer. The phage suspension was vortexed and left at room temperature for 15 minutes. A 0.3 mL aliquot of the phage solution was added to 0.25 mL of appropriate host cells. Phage and cells were allowed to adsorb at room temperature for 15 minutes. Molten top agar (3 mL) was added to the phage-cell mixture, which was immediately poured onto a fresh TB plate, and incubated upright at 37°C for 5-8 hours. A cell only plate was also incubated as a control (0.25 mL host cells and 3 mL top agar). Once the developing plaques have destroyed most of the cell lawn, a sterile glass spreader was used to scrape the top layer off of the plate into a sterile 30 mL Corex glass centrifuge tube. The plate was washed with 3 mL TM buffer, which was also added to the Corex tube. A few droplets of chloroform were added to the tube, which was vortexed hard and let sit at room temperature for 15 minutes. The tube was spun at 10,000 rpm for 15 minutes and the supernatant was collected and stored at 4°C

for future use.

2.3.12.3. High Titer CI^+ Lysates

High titer λcI^+ lysate preparations are difficult to obtain, due to the high lysogenization frequency of the phage. The basic lysate procedure was modified in order to increase the titer of CI^+ phage preparations. Two strip streaked plaques of the CI^+ phage to be produced were added to 0.5 mL of TM buffer with 10 µL chloroform, vortexed well and incubated at 37°C for 5 minutes. Then, 0.3 mL of the phage suspension (ensuring no chloroform is picked up) was added to 0.1 mL of fresh overnight host cells and 0.3 mL of 0.01 M $MgCl_2/CaCl_2$ and held at 37°C for 10 minutes to allow cells and phage to attach. The mixture was added to 15 mL of prewarmed TB and incubated with vigorous shaking at 37°C for 4.5-5.5 hours. Chloroform was added and shaking was continued for 30 minutes. The lysate was centrifuged at 10,000 rpm for 10 minutes and the supernatant was collected and stored at 4°C.

2.3.13. Lysogen Construction

Fresh overnight stationary phase host cells (0.3 mL aliquot) were mixed with 3 mL of molten TB top agar and poured onto a TB plate. Once solidified, 0.02-0.05 mL of the desired phage lysate was added to the center of the plate. The phage spot was allowed to dry and the plate was incubated overnight at 30°C. The next day, there was an area of partial lysis where the phage spot was added. Cells within this region were streaked to a fresh plate for single colonies. Several single colonies (possible lysogens

or λ-resistant cells) were tested for λ immunity by cross streaking or functional immunity assays, as described below, in sections 2.3.14. and 2.3.15.

2.3.14. Cross Streaking Immunity Assay

The phage strains to be used as test phage were streaked across a plate in a straight line and allowed to dry. Unless otherwise indicated, the test phages are usually λ*cI*72 and λvir. The cells to be tested for immunity were streaked across the phage in a perpendicular direction, ensuring that a small inoculum of cells was utilized. Cross streaking plates were incubated at 30°C unless otherwise indicated. Cells without λ immunity, *i.e.* sensitive cells, will be infected by the phage as the cell line crosses the phage line, and will be lysed in the region where the cells and infecting phage interact, as a result of vegetative phage growth within the infecting cells. Cells with λ immunity will be able to grow past the λ*cI*72 line, but will be infected by λvir. However, neither λ*cI*72 nor λvir will be able to infect host mutants to which λ phages cannot attach. The test cells were also streaked to master 30°C and 42°C plates to test for cells with a temperature sensitive prophage, which are lysed when grown at 42°C.

2.3.15. Functional Immunity (FI) Assay

A fresh overnight stationary phase culture of W3350(λ*imm*434T) host cells (0.3 mL aliquot) was mixed with 0.1mL of a high titer (>1 X 10^9 pfu/mL) lysate of phage λ*imm*434*cI* plus 3 mL of molten TB top and the mixture was poured onto a TB plate and allowed to solidify. Cells to be tested for λ immunity were stabbed onto the

plates using sterile toothpicks. Plates were incubated overnight at 30°C. Host cells containing an *imm*λ prophage will be able to homologously recombine with the free λ*imm*434*cI* phage in the overlay, producing an *imm*λ phage recombinant, capable of infecting the W3350(λ*imm*434T) cells in the overlay, causing areas of lysis around the stab.

2.4. METHODS UTILIZED IN THE P-INTERFERENCE STUDY

2.4.1. P-killing Transformation Survival Assay

Cells were transformed with the P^+ or P^- plasmids at 30°C as described in section 2.3.3. Transformations were repeated at 34°C, 37°C and 42°C.

2.4.2. Isolation of Spontaneous pHB30^{nl-42} Transformants

GM2932 cells containing a *mutH* mutation were transformed with pHB30 DNA at 30°C, as described in section 2.3.3. Representative GM2932[pHB30] transformants were streaked to fresh TB+Amp plates. The plates were incubated at 30°C for 4 hours to allow cell and plasmid replication to occur, then were shifted to 42°C and incubated overnight. Visible colonies were streaked to fresh TB+Amp plates and tested for their ability to grow at 30°C and 42°C. The plasmids were isolated from transformants able to grow at 42°C. The newly isolated plasmids, now termed pHB30^{nl-42} (non-lethal at 42°C), were transformed into the wild type strain, W3350. W3350[pHB30^{nl-42}] cells were tested for P activity in the Complementation-Marker Rescue assay (see below).

2.4.3. Complementation-Marker Rescue Assay

An aliquot of stationary phase cells (0.25 mL) to be tested for O or P activity was mixed with 0.1 mL of diluted λ*imm*434*O*am205 or λ*imm*434*P*am3 phage (approximately 1000 pfu) and 3.0 mL top agar. The mixture was poured onto a TB plate and incubated overnight at 34°C. The resulting plaques were counted. At 34°C the λ*cI*[ts]857 repressor, present in pHB30, is leaky, and permits transcription from the p_R promoter to occur at a low level. This transcription will permit the expression of downstream genes, *i.e.*, the *cro-O* fusion gene, *P* and *ren*. Infecting phage containing amber mutations in essential genes are unable to grow on wildtype host cells. However, if the host cell is producing the phage protein (the same protein that is defective in the infecting phage), the infecting phage can use the host-encoded protein to grow and produce a plaque.

Amber mutations spontaneously revert to wild type at a frequency of approximately 1 X 10^{-7}. Because only 10^3 phage particles were plated, no wild type revertants are expected to develop. Because the amber mutant phage is unable to grow efficiently in a *sup*E$^-$ host strain, 0 pfu are expected in a host strain without pHB30. Cells containing wild type pHB30 growing at 34°C produce a Cro-O fusion protein and a functional P protein. Thus, an infecting heteroimmune phage containing an amber mutation in the *P* gene should be able to grow efficiently in a host containing wild type pHB30 (*i.e.* due to complementation in trans by the plasmid for the defective *P* on the infecting phage), and full complementation should enable the appearance of approximately 1000 plaques per assay plate. In contrast, O complementation should not be possible (pHB30 should not produce a functional O

protein for use by the infecting phage). However, because the plasmid contains some *O* gene sequence, marker rescue recombinational exchange between the mutated *O* gene of the infecting phage and the wild type portion of the *O*-fragment on the plasmid could yield some O^+ recombinant phages, which when released from the infected cell would form a plaque on the non-suppressing host cells in the top agar medium. Mutant pHB30 plasmids, able to be transformed at 42°C, can be tested by recombination for the presence of the *O*-fragment and *P* gene, and by complementation for the expression and activity of these gene products in one step, using this simple genetic assay.

2.4.4. Plasmid Loss Assay

I decided to see if cells expressing *P* had increased rates of plasmid loss compared to cells without *P* expression. To do this, cell titers were assayed with and without selection to maintain the plasmid (*i.e.* cells grown in the presence or absence of ampicillin); and with and without *P* gene expression (*i.e.* cells containing pHB30(P^+) or pHB31(P^-), grown at 34°C or 30°C).

Cells containing pHB30 or pHB31 were grown overnight to stationary phase in TB+Amp at 30°C. Aliquots of the culture cells were removed, diluted and plated on TB or TB+Amp plates at 30°C or 34°C. Following incubation for 16 hours, resultant colonies were counted. The fraction of cells retaining the plasmid was measured as cell titer on TB+Amp / cell titer on TB. The data at 34°C was compared to the data at 30°C.

2.4.5. P-killing Transient Induction Survival Assay

Cells were transiently exposed to P protein for up to 5 hours and cell viabilities were determined. Fresh overnight cultures (stationary phase) to be assayed were diluted into fresh TB (with ampicillin where required) and grown up to log phase (A_{575nm} of approximately 0.1-0.15) at 30°C. At time 0 (A_{575nm} ~0.1-0.15), the cultures were swirled in a 50°C water bath for 15 seconds and incubated at 42°C. Every 30 minutes, absorbance readings (A_{575nm}) were taken; and every 60 minutes, a culture aliquot was removed that was used for dilution and plating to determine cell viabilities. Cell viability was measured as the titer of the cell aliquot (cfu/mL) / the cell titer determined for the culture aliquot removed at time 0 (cfu/mL).

2.4.6. Gram Staining

Cells were gram stained in order to provide contrast for visualization of cell morphology under a light microscope. A small volume of cell culture (10 μL) was smeared onto a microscope slide and allowed to air dry. The cells were heat fixed by passing through a flame briefly. The slide was flooded with Crystal Violet dye (crystal violet, saturated alcoholic solution 10 mL; ammonium oxalate 1% aqueous solution 40 mL) for 1 minute and washed with water. The slide was then flooded with Grams Iodine (iodine 1 g; potassium iodide 2 g; distilled H_20 300 mL) for 1-2 minutes and rinsed with water. The slide was rinsed with acetone and immediately washed with water. The slide was counterstained with Safranin dye (safranin, 3.4% alcoholic solution; diluted 1/10 in H_20) for 1 minute, washed and blotted dry. The stained slide was then analyzed under a light microscope (100X magnification with oil immersion)

in order to visualize relative cell length for comparative qualitative measurements of cellular filamentation.

2.5. METHODS UTILIZED IN THE INHIBITION PHENOTYPE STUDY

2.5.1. OOP Phenotype CII Inactivation Assay

The over-expression of OOP RNA from a plasmid has been demonstrated to inhibit CII expression (Takayama *et al,* 1987), causing infecting phage to produce clear plaques. An aliquot (0.3 mL) of stationary phase cells to be tested for OOP activity (*i.e.* 594[pHB27R]) were mixed with 0.1 mL of diluted λ*cI*857(*18,12*)P22 phage and 3 mL of warm TB top agar and poured onto TB plates. [Note: the *rep*P22 phage λ*cI*857(*18,12*)P22 was used here, as it was insensitive to the Inhibition Phenotype, to be discussed in further detail in the results section]. The plates were incubated at 30°C overnight. Plaque morphology was then determined to be clear (OOP^+) or turbid (OOP^-).

2.5.2. Inhibition Phenotype Plating Assay

*Rep*λ and *rep*P22 infecting phages were plated on several plasmid-containing host cell strains (*i.e.* 594[pHB27R]) in order to measure plasmid-mediated inhibition of phage plating. An aliquot (0.25 mL) of a fresh overnight cell culture was mixed with 3 mL of warm TB top agar and 0.1 mL of diluted *rep*λ or *rep*P22 phage lysate, and poured onto TB or TB+Amp plates. Plates were incubated at 30°C overnight and plaques were counted. The results were expressed as efficiency of plating, *i.e.* phage

titer on 594[test plasmid] (pfu/mL) / phage titer on 594 (*i.e.* plasmid free cells) (pfu/mL).

2.5.3. Inhibition Phenotype Plaque Size Assay

Phage plaque sizes were determined on the *E. coli* host 594 cells that had been transformed with plasmids containing various λ fragments. The plaque size was measured using a tissue culture (inverted) microscope at 4 X magnification with an eyepiece grid. Each grid interval was 0.45 mm at 4 X magnification. The phage plaque diameters were measured as grid units, and plaque diameter was scored as the number of grids/plaque. Approximately 30 plaques were measured for each of the phages plated on each of the transformed cells and the average plaque diameter and standard error were determined for each of the individual infections. All of the measurements taken for a given phage were performed in parallel on each one of the host strains.

2.5.4. Inhibition Phenotype Prophage Induction Assay

*Rep*λ or *rep*P22 prophages from lysogenic cells transformed with plasmids containing various λ fragments were induced in order to test the ability of the plasmids to interfere with the induction process. Lysogenic cells in which the prophage was to be thermally induced were grown up in 20 mL TB (with ampicillin where required) with shaking at 30°C to an A_{575nm} of ~ 0.15. At time 0 (*i.e.* at $A_{575nm} = 0.15$) the prophage within the culture cells was induced by swirling the culture flask in a 50°C water bath for 15 seconds and then transferring it to a 42°C shaking water bath for 7

hours. Absorbance readings (A_{575nm}) were taken at 30 minute intervals. Each assay was separately repeated. The results were averaged, with standard errors being calculated.

2.5.5. Phage Burst Assay

Host cells transformed with plasmids containing various λ fragments were infected with a *rep*λ or a *rep*P22 phage at a high or low moi. The number of phage particles released per infected cell (*i.e.* phage burst) was compared for each phage, at high and low moi. The assay was done in order to determine if the λ plasmids were interfering with *ori*λ-dependent DNA replication initiation; which can be bypassed in multiply infected cells (Hayes, unpublished data). A fresh overnight culture of cells (grown at 30°C in TB+Amp where required) was washed and resuspended in Φ80 buffer. An aliquot (0.1 mL) of the washed host cells was mixed with 0.2 mL of 0.01 M $MgCl_2/CaCl_2$ (on ice) with an appropriate volume of phage in order to obtain an moi of either 5 or 0.01. The infection mix was held on ice for 15 minutes to allow for phage attachment. The assay mixture was then transferred to 42°C (the beginning of the incubation step was set to "0-time") and incubated for 10 minutes. The mixture was spun in a Tabletop centrifuge for 1.5 minutes and the pellet was resupended in Φ80 buffer. This wash step was repeated twice. The last resuspension was done using prewarmed 42°C TB to produce a final volume of 0.4 mL. The 0.4 mL infection mixture was divided in half. One 0.2 mL aliquot was used to proceed with the infection protocol. The second 0.2 mL aliquot was centrifuged and used to measure the number of starting cells and the number of contaminating free phage.

The first 0.2 mL aliquot of the infection mix was used to inoculate 20 mL of TB (+ Amp where required), which was then incubated with shaking at 42°C. At 65 and 110 minute intervals, aliquots from the culture infection were removed, diluted and plated to determine the phage titer of the lysate. The number of phage particles released per 0.2 mL aliquot was calculated.

The remaining 0.2 mL aliquot from the infection mixture was spun once more and the supernatant was removed to a fresh tube. The number of free phage per 0.2 mL aliquot was calculated from this supernatant. The pellet was resuspended in 0.2 mL of prewarmed TB, diluted and plated for phage, in order to determine the number of infected centers present in the culture infection. The number of infective centers per 0.2 mL aliquot was calculated. The pellet aliquot represents the infective centers, which are the cells infected by phage and have not yet lysed. Calculating the number of infective centers, rather than using the number of starting cells, gives a more accurate phage burst calculation, as not every cell present will be infected. The plating of infective centers must be completed within 25 minutes of time 0, before the infective centers begin to burst.

-Phage Burst was calculated as the Total # of phage released / # of Infective centers.
-The Infection Efficiency was calculated as the # of Infective Centers / the # of Starting Cells.

-In both calculations, the number of contaminating free phage were taken into consideration.

Sample phage burst calculation for 594[pBR322] cells infected with λ*cI*857 at an moi of 5:

1. Number of starting cells at time 0.

-Cell titer = 1.38×10^9 cfu/mL

-0.1mL of cells was added to the 0.4 mL infection assay reaction volume

- 1.38×10^9 cfu/mL X 0.1 mL

= 1.38×10^8 cfu / 0.4 mL infection reaction volume

2. Phage Released 65 minutes after infection (from 0.2 mL infection aliquot diluted into 20 mL TB and grown at 42°C for 110 minutes).

-Phage titer = 4.90×10^7 pfu/mL

-Number of phage released from the original 0.2 mL aliquot in the infection reaction mix = phage titer X Dilution factor of 100 X 0.2 mL = 4.90×10^7 pfu/mL X 100 X 0.2 mL

= 9.80×10^8 pfu / 0.2 mL infection aliquot

3. Number of Infective centers (Number of cells infected by phage) from resuspended pellet.

-Phage titer from resuspended pellet = 1.69×10^8 pfu/mL

-Number of infective centers per original 0.2 mL infection aliquot

= 1.69×10^8 pfu/mL X 0.2 mL

= 3.38×10^7 pfu / 0.2 mL resuspended pellet

4. Number of free phage present in the supernatant of the spun 0.2 mL infection aliquot.

-Phage titer from supernatant = 4.75×10^6 pfu/mL

-Number of free phage per 0.2 mL infection aliquot

= 4.75×10^6 pfu/mL X 0.2 mL

= 9.50×10^5 pfu / 0.2mL supernatant

5. Infection Efficiency (calculation of how many cells were infected)

-[(Number of infected centers / 0.4 mL reaction mix) – (Number of free phage per 0.4 mL reaction mix)] / [(Number of starting cells / 0.4 mL reaction mix) – (Number of free phage per 0.4 mL reaction mix)]

= [2(3.38×10^7 pfu / 0.2 mL) – (9.50×10^5 pfu / 0.2 mL)] / [1.38×10^8 cfu / 0.4 mL]

= 0.48 = 48% of the starting 594[pBR322] cells were infected by λ*cI*857

6. Phage Burst (Number of phage particles released per infected cell)

-[(Number of phage released per 0.2 mL aliquot) – (Number of free phage per 0.2 mL aliquot)] / [(Number of infective centers per 0.2 mL aliquot) – (Number of free phage per 0.2 mL aliquot)]

= [(9.80×10^8 pfu) – (9.50×10^5 pfu)] / [(3.38×10^7 pfu) – (9.50×10^5 pfu)]

= 27.15 phage released per infected cell

2.5.6. Assay for Measuring Survivor Frequencies After Phage Infection

OOP RNA has been demonstrated to inhibit CII activity, and thus interfere with the process of lysogenization (Takayama *et al*, 1987). To assay this, the survival frequency after phage infection of cells transformed with plasmids containing λ fragments was measured. An aliquot (0.2 mL) of fresh overnight stationary phase cells (*i.e.* 0.2 mL represents ~4.0 X 10^8 cells of an overnight culture grown up to 2.0 X 10^9 cfu/mL) was mixed with 0.3 mL 0.01 M $CaCl_2/MgCl_2$ and 0.1 mL of λ*cI*857 at an moi of 2 and incubated at 37°C for 15 minutes to allow adsorption. The mixture was diluted 1/100 in TB and incubated with shaking at 30°C for 75 minutes. The cells in the infection mixture were pelleted at 6,000 rpm for 6 minutes, washed once in 20 mL TB, resuspended in 1 mL TB, and then diluted in Φ80 buffer and plated on TB (+Amp where required) overnight at 30°C. The colony titer was used as a measurement of the efficiency of cell survival from the λ*cI*857 infection and for determining the frequency of lysogenization. The colonies surviving λ*cI*857 infection were replica plated to plates seeded with approximately 1 X 10^8 λ*cI*72 pfu and incubated overnight at 30°C. Predominantly, only lysogenic cells will be able to form colonies on the plate spread with CI^- phage. The infection survival frequency was calculated as the cell titer after infection divided by the starting cell titer.

2.5.7. Isolation of SIP Mutants

λ*cI*857 was plated on 594[pHB27R] at 30°C at low dilution (10^{-1}-10^{-4}). Essentially, an aliquot of host cells (0.25 mL) was mixed with 0.1 mL of a λ*cI*857 diluted from 10^{-1}-10^{-4} and 3 mL of molten top agar. The entire mixture was poured

onto a TB plate and incubated overnight at 30°C. A visible plaque was stabbed with a sterile toothpick and swirled into 10 μL of TM buffer. The phage suspension was streaked onto a fresh overlay of 594[pHB27R] using sterile paper strips, and incubated overnight. The larger plaques that formed on the plate were re-picked, mixed with host cells and incubated for new plaques. It was noticed that there were always plaques of various sizes in the areas of lysis (most phage lysates produce plaques of uniform size). So, the purification steps were repeated a total of 13 times in an effort to produce lysates containing plaques of uniform size. Ultimately, all lysate preparations retained the multiple plaque size phenotype when plated on 594[pHB27R] host cells. After the 13 enrichment cycles, 10 independent λ*cI*857 SIP lysates (**S**uppress **I**nhibition **P**henotype), able to plate on 594[pHB27R] at high efficiency, were produced.

2.5.8. Genetic Assays for SIP Phage Characterization

2.5.8.1. Plating assays

One hypothesis for the ability of the SIP phages to escape the Inhibition Phenotype is that they have integrated the pHB27R plasmid into their genomes and are replicating from the ColE1 origin, independently of *ori*λ. The ten SIP phages were plated on host strains interfering with either *ori*λ or ColE1-dependent replication initiation. If the phages contain two functional origins of replication (ColE1 from pHB27R and *ori*λ from λ), then blocking replication from one origin should not prevent phage replication. The *rep*λ phage λ*cI*857, the parental phage that the SIP

phages were derived from, is unable to produce a plaque on the host strain 594 *dnaB*grpD55 because the mutated *dnaB* allele is non-functional for *ori*λ-dependent replication initiation. A ColE1 plasmid will not be able to replicate in 594[pHB27] host cells, due to ColE1 incompatibility. The Rop protein produced by pHB27 stringently regulates plasmid copy number, preventing the replication of an incoming ColE1 origin. If the SIP phages do contain two functional origins, they should be able to plate and produce plaques on both of these host strains. To test this hypothesis, 0.1 mL of each diluted SIP phage lysate was mixed with 0.3 mL of each of the two host strains, added to 3 mL of molten top agar and poured onto TB plates, which were then incubated overnight at 30°C. Resulting plaques were counted and plating efficiencies were calculated for each SIP mutant on each of the host strains.

2.5.8.2. Assaying SIP Lysogens for Ampicillin Resistance

594(λ*cI*857) lysogenic cells are not able to grow in the presence of ampicillin. If the SIP phages have integrated the pHB27R plasmid into their genome, they should be able to express the Amp^R gene from pBR322, and thus should be able to grow on TB+Amp plates. To test this hypothesis, nonlysogenic 594 culture cells were infected with wild type λ*cI*857 and with the 10 SIP phage lysates, as described in section 2.5.1, in order to lysogenize them. The lysogenic survivor cells were streaked to fresh TB and TB+Amp plates and incubated for 48 hours at 30°C. The ability of the 594(λ*cI*857) and 594(SIP) cells to grow in the presence of ampicillin was determined.

CHAPTER 3. RESULTS

3.1. EFFECT OF λ *P* EXPRESSION ON *E. COLI* HOST CELLS

3.1.1. Background Data / Rationale for Study

Early studies on P protein over-expression in *E. coli* cells demonstrated that high levels of P protein interfered with host cell metabolism (Klinkert and Klein, 1979). More recent studies (Maiti *et al*, 1991a) showed that the effect of P protein on host cells was more severe, in fact being lethal to the majority of exposed cells. The apparent discrepancy between these findings begged further study. Many questions regarding P protein-host interactions remained. What exactly is going on inside cells exposed to high levels of P protein? Is P protein over-expression lethal or not? What is the cellular target(s) for P? Can we find host mutants resistant to P? Can we find P protein mutants unable to produce the "P-effect" in host cells? Is the "P-effect" reversible? Is P degraded in the cell? These are the questions I attempted to find answers for, using a genetic approach.

3.1.2. Hypothesis for the Effect of *P* Expression on Host Cells

It has been shown that λ *P* expression can be lethal to host cells (Maiti *et al,* 1991a), termed 'P-killing' or 'P-lethality'. My hypothesis, based on previous results

(Klinkert and Klein, 1979; Bull, 1995), is that "P-lethality" is due to the sequestering of DnaB by λ P, and that this P-effect is not necessarily lethal.

3.1.3. P-killing Transformation Assay

The 1991 study by Maiti *et al.* utilized a transformation assay as a system for measuring P-lethality. Basically, several different plasmid constructs were transformed into host cells and survivor colonies were counted. Using this system, Maiti *et al.* deduced that *P* over-expression was lethal to host cells, based on a finding that plasmids over-producing P protein produced no survivor colonies upon transformation. The plasmid constructs used by Maiti *et al.* produced P protein constitutively. I decided to test their findings using constructs in which I could regulate *P* expression levels.

3.1.3.1. Plasmid pHB30

The plasmid pHB30 (described in section 2.1.3.1.1 and illustrated in Fig. 3.1), which was constructed by Harold Bull (1995), expresses λ *P* from the p_R promoter, which is negatively regulated by the λ repressor expressed from gene *cI*. pHB30 carries a temperature sensitive allele of *cI*, *cI*[ts]857, which permits the modulation of transcription from p_R by varying the temperature of the cells. At 30°C, the λ CI repressor is active, preventing transcription from p_R. At 34°C, the binding of CI becomes unstable. As the temperature increases, repressor binding to the operator sites diminishes, allowing transcription from the p_R promoter to produce λ *P* gene product (Slavcev and Hayes, 2003).

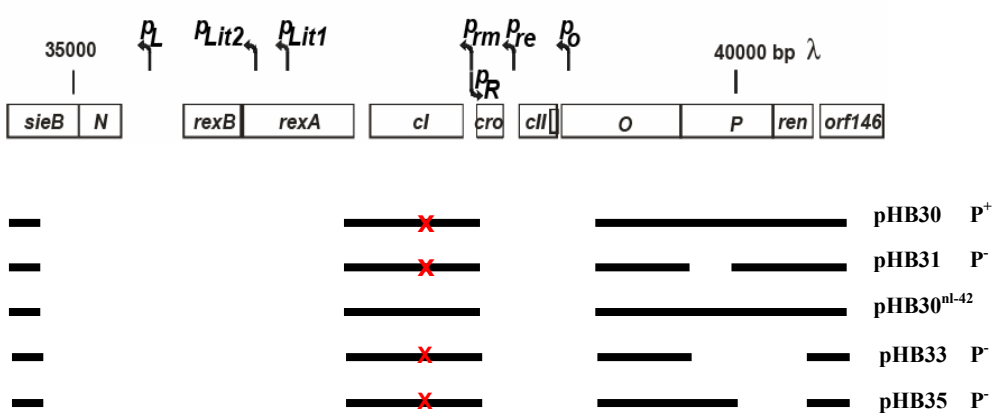

Figure 3.1. P-Interference Plasmid Maps. All plasmids are pBR322/λ hybrids. The λ DNA is represented by thick black bars. **X** represents the relative position of the 857[ts] mutation mapping 198 bp from the N-terminal end of the 710 bp *cI* gene. pHB30, pHB31, pHB33 and pHB35 were constructed by Harold Bull (1995). pHB30^{nl-42} was isolated during the current study (See section 3.1.4); pHB30^{nl-42} contains a point mutation within *cI* which reverts the *cI* ts allele back to wild type, preventing the expression of downstream genes at all temperatures.

Table 3.1. Transformation Assay for Studying the Effect of λ *P* Expression on Host Cell Survival

Transforming Plasmid	Plasmid Transformation of 594 Cells [a]			
	30°C	34°C	37°C	42°C
pHB30	10^{-7}-10^{-8} [b]	10^{-7}-10^{-8}	$<10^{-8}$ [c]	$<10^{-8}$
pHB31	10^{-5}-10^{-6}	10^{-5}-10^{-6}	10^{-5}-10^{-6}	$<10^{-8}$
pHB33	10^{-4}-10^{-5}	10^{-4}-10^{-5}	10^{-4}-10^{-5}	10^{-4}-10^{-5}
pHB35	10^{-4}-10^{-5}	10^{-4}-10^{-5}	10^{-4}-10^{-5}	10^{-4}-10^{-5}

[a] *E. coli* 594 cells were transformed with plasmids pHB30, pHB31, pHB33 or pHB35 at 30°C, 34°C, 37°C or 42°C as described in section 2.4.1.
[b] Results are presented as Transformation Efficiency:
(number of Amp^R transformant cells / number of total survivor cells)
[c] A transformation efficiency of $<10^{-8}$ means that no Amp^R cfu were seen when 0.1 mL of cells were spread on TB+Amp plates at a dilution of 10^{-1}.

Transformation efficiencies of pHB30 in the wild type host strain 594 were assayed at 30°C, 34°C, 37°C and 42°C, Table 3.1. In bacteriophage λ development, CI is initially made from the p_{RE} promoter, activated by CII. Once CI levels reach a threshold level, p_R transcription is turned off, leading to a decrease in CII and subsequent p_{RE} transcription of the *cI* gene. At this point, CI is produced via transcription from p_{RM} and CI levels in the cell remain high. In pHB30, the *cII* gene is deleted, so no transcription from p_{RE} occurs. At 30°C, CI repressor concentration from the weak p_{RM} promoter will slowly build up in cells containing pHB30. Until CI reaches its threshold level, p_R transcription will occur. At higher temperatures, the repressor formed (*cI*[ts]857) is progressively less effective, and is fully inactive at 42°C. The pHB30 transformation efficiency (number of transformants/number of survivor cells) at 30°C was found to be extremely low (10^{-7} to 10^{-8}), Table 3.1. A similar transformation efficiency was seen at 34°C. No transformants were ever detected at 37°C or 42°C, where *P* expression is expected to be high. These results suggested that high levels of gene expression from the plasmid pHB30 are detrimental to host cells.

3.1.3.2. Plasmids pHB31, pHB33 and pHB35

In order to confirm that the transformation lethality of pHB30 was dependent upon the expression of *P* downstream from p_R, three pHB30 *P* deletion derivatives, constructed by Harold Bull (1995), described above in section 2.1.3.1.1 and illustrated in Fig. 3.1, were tested for transformation efficiencies at the four temperatures.

pHB31 contains an in-frame, N-terminal *P* gene deletion of 78 codons. This

plasmid construct produced transformants at an efficiency of 10^{-5} to 10^{-6} (*i.e.* 100 X higher than pHB30) at 30°C, 34°C and 37°C; no transformants were seen at 42°C, Table 3.1. These results suggested that the effect seen in pHB30 was indeed due to P protein over-expression. It seems that the pHB31 P fragment is also detrimental to host cells when fully expressed at 42°C.

pHB33 contains a large deletion removing all but the first eight codons of the 233 codons of gene *P* and all of gene *ren*. The fragment potentially translated from the *P-ren* deletion produces an 18 aa protein product (eight codons from the *P* gene N-terminus and ten codons downstream of gene *ren*) before encountering a TGA stop codon at λ40642. pHB33 transformed at frequencies of 10^{-4} to 10^{-5} at all temperatures, Table 3.1, suggesting that P protein over-expression is responsible for the effect seen from pHB30.

pHB35 contains a large deletion removing the C-terminal 95 codons of gene *P* and all of gene *ren*. The fragment potentially translated from the *P-ren* deletion produces a protein consisting of the first 138 aa of P fused to an additional 15 aa downstream of gene *ren* before a TGA stop codon is encountered at λ40661. pHB35 transformed at frequencies essentially identical to pHB33, of 10^{-4} to 10^{-5} at all temperatures, Table 3.1, suggesting that the carboxy terminal, rather than the amino terminal, end of the *P* gene is responsible for the negative influence of P protein on cell metabolism, and that P protein over-expression is responsible for cell lethality produced by the transformation of cells with pHB30 at temperatures where CI cannot be made or is not active.

The actual DNA concentrations of the plasmid preparations used were not

known. The transformation efficiencies of the four plasmids constructs cannot be directly compared without normalizing the amount of DNA used. However, the transformation efficiencies at each of the four temperatures per plasmid can be directly compared. These results demonstrate that pHB30 is the only plasmid that increasingly inhibits the development of Amp^R colonies as the temperature increases; suggesting that *P* expression is responsible for this effect.

3.1.3.3. Plasmid Loss Assays

Since P-killing was determined by measuring the number of survivor colonies on TB+Amp plates, any cells that survived as a result of plasmid loss would not be detected, *i.e.* they would no longer be Amp^R and thus would not form colonies on TB+Amp plates. It was essential to determine whether λ *P* expression from pHB30 at elevated temperatures influenced plasmid loss levels.

Fresh overnight cultures of stationary phase cells to be assayed (grown up in TB+Amp at 30°C) were diluted and plated on TB and TB+Amp plates at 30°C and 34°C and incubated overnight. At 34°C, the λ*cI*857 repressor's ability to bind the rightward O_R operator sites is less effective than for the CI^+ repressor, and consequently, there is a trace of rightward transcription from the rightward promoter p_R (Slavcev and Hayes, 2003).

The results in Table 3.2 demonstrate that λ *P* expression did increase plasmid loss levels. Plasmid pHB31, phenotypically P^-, was retained in approximately 100% of cells at both 30°C and 34°C. Plasmid pHB30 (P^+) was retained in 74% of cells at 30°C and in only 13% of cells at 34°C. These results suggest that the majority of cells

Table 3.2. Effect of λ *P* Expression on Plasmid Loss Levels

Strain	Cell titer (cfu/mL) [a]					
	30°C			34°C		
	TB Media	TB + Amp Media	% plasmid retention 30°C [b]	TB Media	TB + Amp Media	% plasmid retention 34°C
594[pHB30]	$1.2x10^9$	$8.6x10^8$	74	$7.9x10^8$	$1.0x10^8$	13
594[pHB31]	$1.6x10^9$	$1.6x10^9$	101	$1.4x10^9$	$1.4x10^9$	94

[a] Overnight cultures were grown up in TB+Amp media at 30°C, then diluted and plated overnight on indicated media at indicated temperatures, and cfu were counted. Presented cell titers were rounded to two significant figures; however, three significant figures were used in all calculations.

[b] % Plasmid retention was determined by the cell titer on TB+Amp plates /cell titer on TB plates X 100.

carrying a plasmid expressing the deleterious P protein will lose the plasmid. By carrying out all of my P assays on TB+Amp plates, I ensured that all assayed cells contain at least some element of the Amp^R plasmid construct(s).

3.1.4. Isolation and Characterization of $pHB30^{nl-42}$ Plasmids

P protein, expressed from pHB30 at 42°C, prevents the recovery of colonies surviving transformation at elevated temperatures. I asked whether mutants of pHB30 could be isolated from among rare cell transformants at 42°C. It was hypothesized that these plasmids (termed $pHB30^{nl-42}$ for non lethal at 42°C) would contain mutations that suppressed the lethal constitutive expression of gene *P* and might identify the motif's within P that contribute to its inhibitory influence on cell metabolism.

3.1.4.1. Isolation of $pH30^{nl-42}$ Transformants

GM2932 *E. coli* cells contain a mutation, *mutH53*, defective for MutH activity, an important component of the mismatch repair system. MutH-defective cells have an increased spontaneous mutation frequency.

In preliminary experiments, GM2932 host cells were transformed with pHB30 DNA at 42°C, but no Amp^R colonies developed (transformation efficiency of $< 10^{-8}$, data not shown). The transformation experiment was then repeated at 37°C, with the same results (data not shown).

In order to isolate pHB30 mutants able to be stably maintained in host cells at 42°C, the transformation protocol was altered slightly. GM2932 host cells were transformed with pHB30 DNA at 30°C. GM2932[pHB30] transformants were streaked

to fresh TB+Amp plates and incubated at 30°C for four hours, to allow cell and plasmid replication to occur, and were then shifted to 42°C and incubated overnight. Survivor cells were streaked for single colonies on TB+Amp plates. Of the single colonies surviving transformation, 22 randomly chosen colonies were re-streaked to fresh TB+Amp plates. The plasmids were isolated from the 22 single colonies, and were then transformed, at 30°C, into wild type W3350 cells (W3350[pHB30^{nl-42}]). This step removed the possibility that unselected host mutations were responsible for the survival of the lethal plasmid at 42°C. The new cells were all able to grow at 42°C on TB+Amp plates (data not shown), indicating that the newly acquired ability to be maintained at 42°C was encoded by the plasmid, and not by the host cells.

3.1.4.2. Characterization of pH30^{nl-42} Isolates

pHB30^{nl-42} isolates 1-22 were possibly clonally derived, since they were all obtained from a single transformation event. The plasmid isolates were further characterized using various genetic and molecular biology techniques. Table 3.3 summarizes the results from the various assays.

3.1.4.2.1. Complementation-Marker Rescue Assay

Isolates were tested for P activity by their ability to complement a λ*imm*434*Pam3* infecting phage. SupE$^-$ cells (phage with amber mutations are able to grow on host strains containing suppressor mutations in *tRNA* genes, called SupE$^+$ cells) containing the plasmid to be tested for P activity were mixed with approximately 1000 pfu of λ*imm*434*Oam205* or λ*imm*434*cI⁻Pam3* phage and incubated overnight at 34°C (at

Table 3.3. Summary of pHB30^{nl-42} Isolate Characterizations

Plasmid Isolate	*O*am8 C' or MR [a] at 34°C	*P*am3 C' or MR at 34°C	Detection of Insertions or Deletions via PCR	*P* Sequence Data	p_R/o_R Sequence Data	CI Activity At 42°C	*cI* Sequence Data
pHB30 wt	MR [b]	C'	None [c]	wt	wt	No	*cI*[ts]857
pHB30^{nl-42} #1	None	MR	None	N/A [d]	wt	Yes	N/A
pHB30^{nl-42} #2	None	MR	None	N/A	wt	Yes	N/A
pHB30^{nl-42} #3	MR	MR	None	N/A	wt	Yes	N/A
pHB30^{nl-42} #4	MR	None	None	N/A	wt	Yes	N/A
pHB30^{nl-42} #5	None	MR	None	N/A	wt	Yes	N/A
pHB30^{nl-42} #6	MR	MR	None	wt	wt	Yes	N/A
pHB30^{nl-42} #7	MR	None	None	wt	wt	Yes	N/A
pHB30^{nl-42} #8	MR	MR	None	wt	wt	Yes	N/A
pHB30^{nl-42} #9	None	MR	None	N/A	wt	Yes	*cI* wt
pHB30^{nl-42} #10	None	MR	None	N/A	wt	Yes	N/A

pHB30$^{nl\text{-}42}$ #11	MR	MR	None	N/A	wt	Yes	N/A
pHB30$^{nl\text{-}42}$ #12	MR	MR	None	N/A	wt	Yes	N/A
pHB30$^{nl\text{-}42}$ #13	None	None	None	N/A	wt	Yes	N/A
pHB30$^{nl\text{-}42}$ #14	MR	None	None	N/A	wt	No	N/A
pHB30$^{nl\text{-}42}$ #15	MR	MR	None	N/A	wt	Yes	N/A
pHB30$^{nl\text{-}42}$ #16	MR	MR	None	N/A	wt	No	N/A
pHB30$^{nl\text{-}42}$ #17	None	MR	None	N/A	wt	Yes	N/A
pHB30$^{nl\text{-}42}$ #18	MR	MR	None	N/A	wt	Yes	N/A
pHB30$^{nl\text{-}42}$ #19	None	MR	None	N/A	wt	Yes	N/A
pHB30$^{nl\text{-}42}$ #20	MR	MR	None	N/A	wt	Yes	N/A
pHB30$^{nl\text{-}42}$ #21	MR	MR	None	N/A	wt	Yes	N/A
pHB30$^{nl\text{-}42}$ #22	None	MR	None	N/A	wt	Yes	*cI* wt

[a] Heteroimmune infecting phages λ*imm*434O_{am205} or λ*imm*434P_{am3} were used to measure *O* or *P* gene expression, respectively, in the Complementation (C')/Marker rescue (MR) assay (shown in greater detail in Table 3.4).
[b] None = 0 pfu; MR = <10 pfu; C' = >250 pfu
[c] None indicates that no detectable insertion or deletion was seen upon PCR amplification.
[d] N/A indicates that the isolate was not assayed in that particular region.

34°C the λ*cI*[ts]857 repressor allows a low level of p_R transcription). The resulting pfu were counted. Because the hetero-immune amber mutant phage is unable to grow efficiently in a *sup*E$^-$ host strain, 0 pfu are expected to develop in a host strain without pHB30. Cells containing wild type pHB30 growing at 34°C express a Cro-O fusion protein which was not expected to be functional, and a functional P protein. Thus, an infecting hetero-immune phage containing an amber mutation in the *P* gene should be able to grow efficiently in a host containing wt pHB30 (*i.e.* due to complementation of the *Pam3* defective P by the wt P made by the plasmid), and approximately 1000 pfu should be seen. In contrast, O complementation should not be possible (pHB30 should not produce a functional O protein for use by the λ*imm*434 or *Oam205* infecting phage), but because the plasmid contains some *O* gene sequence, marker rescue by the incoming phage should be possible. Thus, a few pfu should be seen upon infection by an *Oam205* phage, due to successful recombination (the recombinant λ*imm*434O^+ phage is able to grow and produce a plaque on the hetero-immune host). Mutant pHB30 plasmids, able to be transformed at 42°C, can be tested for O and P activity using this genetic assay system, as illustrated in Table 3.4. The results are also included in the general summary, presented in Table 3.3.

W3350[pHB30$^{nl\text{-}42}$] isolates 1-22 were assayed using the genetic strategy illustrated in Table 3.4. None of the isolates was able to complement for O or P defects, but marker rescue for *O* and/or *P* occurred in the majority of isolates. The plasmid pHB30 encodes a Cro-O fusion protein, and thus was not expected to complement for O protein. However, since the *Oam205* mutation on the infecting hetero-immune phage maps to the distal end of *O (*Furth, 1978), marker rescue of the *Oam205* mutation should

Table 3.4. Complementation-Marker Rescue Assay for Measuring O and P Protein Activity at 34°C

Host Cells	Number of Developing pfu per ~1000 Initial Infecting Phage Particles [a]	
	λimm434cIΓPam3	***λimm434cIΓOam205***
W3350	0 [b]	0
W3350[pHB30] – wt	750 [c]	2 [d]
W3350[pHB30^{nl-42}] – 1	2	0
W3350[pHB30^{nl-42}] – 2	2	0
W3350[pHB30^{nl-42}] – 3	2	1
W3350[pHB30^{nl-42}] – 4	0	1
W3350[pHB30^{nl-42}] – 5	2	0
W3350[pHB30^{nl-42}] – 6	5	1
W3350[pHB30^{nl-42}] – 7	0	4
W3350[pHB30^{nl-42}] – 8	4	1
W3350[pHB30^{nl-42}] – 9	2	0
W3350[pHB30^{nl-42}] – 10	1	0
W3350[pHB30^{nl-42}] – 11	6	1
W3350[pHB30^{nl-42}] – 12	1	2
W3350[pHB30^{nl-42}] – 13	0	0
W3350[pHB30^{nl-42}] – 14	0	3
W3350[pHB30^{nl-42}] – 15	3	2
W3350[pHB30^{nl-42}] – 16	2	3

W3350[pHB30$^{nl\text{-}42}$] – 17	7	0
W3350[pHB30$^{nl\text{-}42}$] – 18	2	2
W3350[pHB30$^{nl\text{-}42}$] – 19	3	0
W3350[pHB30$^{nl\text{-}42}$] – 20	2	1
W3350[pHB30$^{nl\text{-}42}$] – 21	5	2
W3350[pHB30$^{nl\text{-}42}$] – 22	3	0

[a] Approximately 1000 pfu of the infecting phage (either λ*imm*434*cI Pam3* or λ*imm*434*cI Oam205*) was mixed with 0.3 mL of fresh overnight cells and 3 mL of molten top agar. The mixture was poured onto TB plates and incubated overnight at 34°C. The resultant plaques were quantitated.

[b] The infecting phages contain amber mutations within essential genes. If the infected host cell does not produce the protein that is defective in the phage, no pfu will develop.

[c] If the host cell is producing the protein that is defective in the infecting phage, the phage will be able to use the trans acting protein to develop (*i.e.* Complementation) and most of the 1000 infecting phage will produce a plaque.

[d] If the host cell is not producing the protein that is defective in the infecting phage, complementation will not occur. If the host cell contains at least some of the gene sequence of the defective protein, homologous recombination can occur between the host and the infecting phage, with marker rescue producing a recombinant phage able to grow and produce a plaque. This recombination event is rare, so only a few pfu will develop.

arise, unless the *O* fragment within the plasmid has been altered or deleted. The wild type pHB30 plasmid was able to complement for the *Pam3* defect, demonstrating a P^+ phenotype. None of the tested pHB30^{nl-42} isolates could complement for the *Pam3* defect, and thus they appeared to be phenotypically defective in P; and yet many of the pHB30^{nl-42} isolates retained at least some portion of *P* since they supported marker rescue of the *Pam3* mutation encoded on the infecting heteroimmune phage.

3.1.4.2.2. Sequencing *P* from pHB30^{nl-42} Isolates

The *P* genes from wild type pHB30 and pHB30^{nl-42} isolates #1-22 were amplified by PCR with primers L20 and R19. The primer pair L20 (λ39465-39484) and R19 (λ40588-40571) are expected to amplify a fragment 1123 nt long. The *P* gene fragment from wt pHB30 and pHB30^{nl-42} isolates #6, 7 and 8 were sent out to the NRC-PBI sequencing service along with primers L20 and R19.

The *P* gene was sequenced for both *l*- and *r*- strands and the results for the three pHB30^{nl-42} isolates revealed a wild type *P* gene sequence, identical to that found in the simultaneously sequenced *P* gene present in pHB30, Table 3.3.

3.1.4.2.3. Searching for Insertions in pHB30^{nl-42} Isolates

The Complementation-Marker Rescue assay results indicated that the pHB30^{nl-42} isolates were phenotypically P^-, while the sequencing data indicated that the isolates were genetically P^+. One possibility for disruption of *P* gene expression is the presence of an insertion element, such as IS2, between the p_R promoter and the gene *P* coding sequence (Hayes *et al,* 1988). Both pHB30 and the pHB30^{nl-42} isolates #1-22

were amplified by PCR with primers LMH29 (λ37905-37922) and RMH25 (λ39626-39604). These primers amplify the λ fragment within pHB30 from the C terminal portion of the *cI* gene through the N-terminal region of gene *P*, producing an expected PCR fragment of 1010 nt. Analysis of PCR fragment sizes, Fig. 3.2, showed that no insertions were present between *cI* and *P* in any of the pHB30 isolates, as noted in Table 3.3. *BstE*II digested λ DNA was used as a marker for estimating DNA band sizes. In wt pHB30 as well as in all 22 pHB30$^{nl\text{-}42}$ isolates, a large, well defined PCR band appeared that was smaller than the *BstE*II 1260 nt band, yet larger than the *BstE*II 700 nt band; suggesting a PCR product near the expected band size of 1010 nt. PCR results for pHB30$^{nl\text{-}42}$ isolates #14 and #15 initially showed no DNA bands; however, when repeated, the expected band was seen (data not shown).

3.1.4.2.4. pHB30$^{nl\text{-}42}$ CI Repressor Activity Assay

Since *P* gene expression of the pHB30$^{nl\text{-}42}$ isolates does not appear to be insertionally disrupted, an alternative explanation was needed for the mechanism behind plasmid survival at 42°C. *P* gene expression is regulated by a temperature sensitive CI repressor protein in the pHB30 constructs, preventing P expression at 30°C, and allowing expression at 42°C. If the *cI* gene had been mutated back to the wild type phenotype, then *P* gene expression would be inhibited at all temperatures (*i.e.* at 42°C). Hence, it was decided to assay repressor activity of the pHB30$^{nl\text{-}42}$ isolates at 30°C and 42°C. Host cells containing the plasmids to be assayed were infected with the homo-immune phage λ*cI*857(*18,12*)P22 at 30°C and 42°C and the number of resulting pfu were counted, Table 3.5. On the host strain W3350, λ*cI*857(*18,12*)P22 produced turbid plaques at 30°C

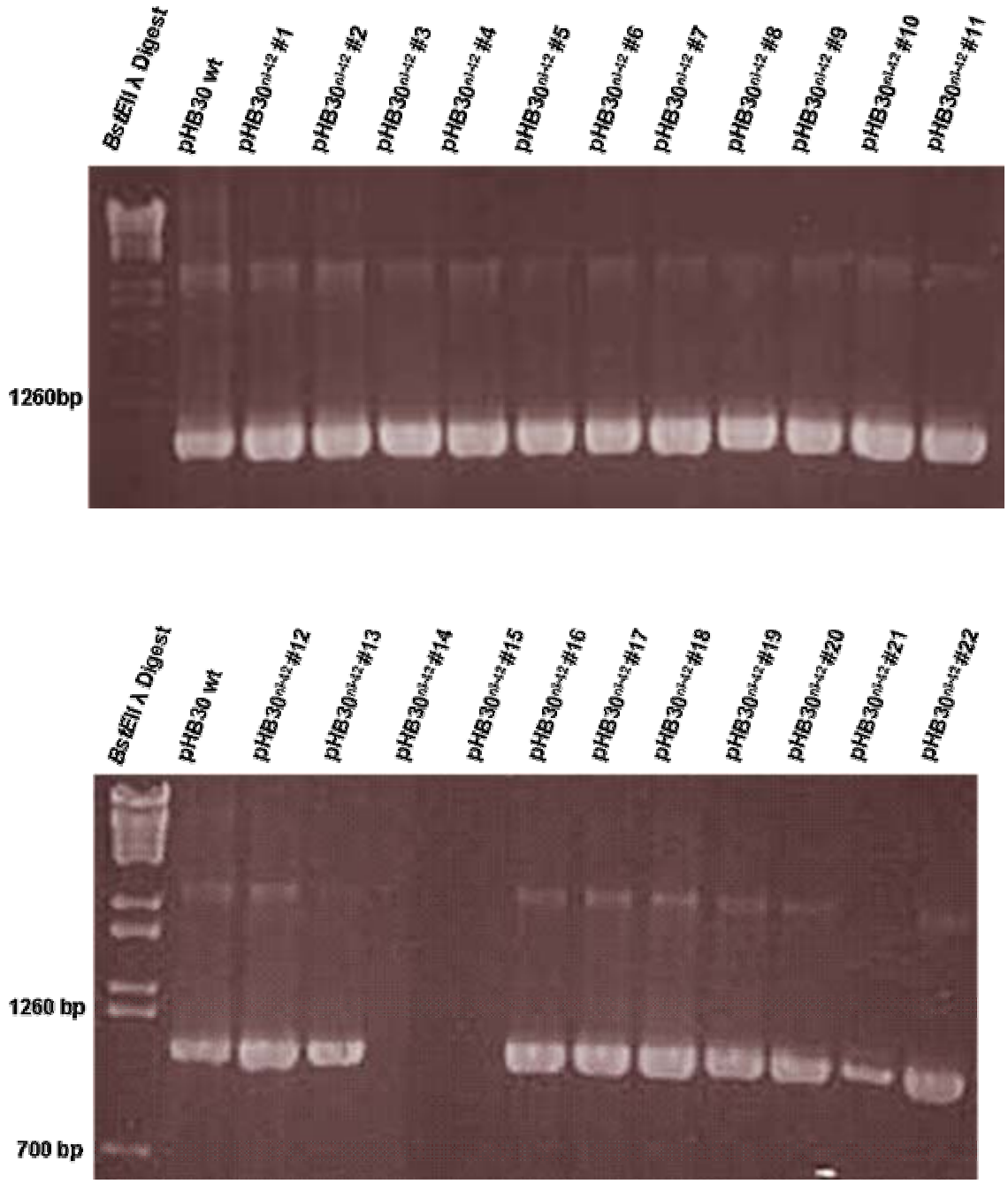

Figure 3.2. Searching for Insertions in pHB30^{nl-42} Isolates. Plasmids, PCR amplified with primers LMH29 and RMH25, were expected to produce DNA bands of 1010 nt. *BstE*II digested λ DNA was used as a marker for estimating band sizes. PCR bands were seen between marker bands 1260 and 700 bp, suggesting that the PCR products were of the expected 1010 size, *i.e.* no insertions or deletions were seen.

Table 3.5. pHB30^{nl-42} CI Repressor Activity Assay

Host Strain	λ*cI*857(*18, 12*)P22 Titer (pfu/mL) and Plaque Morphology [a]	
	30°C	**42°C**
W3350	2.7×10^9 turbid pfu [b]	1.9×10^9 clear pfu [c]
W3350[pHB30]	$< 1.0 \times 10^1$ [d]	No host cell growth [e]
W3350[pHB30^{nl-42}] – #14 and #16	$< 1.0 \times 10^1$	3.7×10^8 clear pfu
W3350[pHB30^{nl-42}] – #1-13, 15 and 17-22	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$

[a] A 0.3 mL aliquot of host cells was mixed with 0.1 mL of λ*cI*857(*18,12*)P22 phage and 3 mL of molten top agar. The mixture was poured onto a TB plate and incubated overnight at either 30°C or 42°C. The resultant plaques were analyzed for plaque morphology (turbid or clear) and phage titer was calculated (pfu/mL).
[b] At 30°C, the CI[ts]857 repressor is active and the presence of lysogens causes plaques to appear turbid.
[c] At 42°C, the CI[ts]857 repressor is inactive and the absence of lysogens causes plaques to appear clear.
[d] In cases where the phage titer is $< 1.0 \times 10^1$, plaque morphology could not be determined, due to the absence of visible plaques.
[e] W3350[pHB30] cells do not grow well enough to produce a viable cell lawn at 42°C, due to the P-killing effect.

(CI^+) and clear plaques at 42°C (CI^-). λ*cI*857(*18,12*)P22 was unable to produce plaques on W3350[pHB30] at 30°C, because the CI[ts]857 repressor made by the pHB30 plasmid binds to the operator sites of the incoming phage, preventing lytic phage development (*i.e.* homo-immunity). λ*cI*857(*18,12*)P22 was unable to produce plaques on $pHB30^{nl\text{-}42}$ isolates #1-13, 15, and 17-22 at 30°C or 42°C, suggesting that the CI repressor was active at both temperatures. The $pHB30^{nl\text{-}42}$ isolates #14 and #16 (Table 3.3 and Table 3.5) retained the *cI*[ts]857 phenotype (*i.e.* CI^+ at 30°C and CI^- at 42°C); the reason for their survival at 42°C remains to be determined (as described further below).

3.1.4.2.5. Sequencing *cI* from $pHB30^{nl\text{-}42}$ Isolates

The *cI* gene from pHB30 and $pHB30^{nl\text{-}42}$ isolates #9 and #22 were amplified by PCR using primers LCI-1 (λ37189-37206) and RCI-1 (λ37996-37979) to produce a PCR product of 807 nt. These PCR fragments were sent to NRC-PBI to be sequenced with primers LCI-1 and RCI-1. The isolates #9 and #22 were each found to contain a true reversion of the *cI*[ts]857 mutation at position 37742 (*i.e.* T to C), converting it back to cI^+ (Table 3.3); whereas pHB30 retained the initial *cI*[ts]857 mutation.

3.1.4.2.6. Sequencing of the p_R/o_R Region of $pHB30^{nl\text{-}42}$ Isolates

The $pHB30^{nl\text{-}42}$ isolates #14 and #16 showed *cI*[ts]857 activity and had no insertions between p_R and gene *P*. One obvious possibility for the inability of these isolates to produce functional P protein at 42°C was that they contained mutations inactivating the p_R promoter, and hence preventing transcription of gene *P*. pHB30 and $pHB30^{nl\text{-}42}$ isolates #14 and #16 were amplified by PCR and sequenced with primers

LMH29 (λ37905-37922) and R11 (λ38913-38897) to amplify the p_R region of the plasmids. The expected PCR fragment was 297 nt. It was found that isolates 14 and 16 contained wildtype lambda DNA sequence throughout the amplified region.

During construction of pHB30, one of two possible *Bgl*II sites 60 nt apart was used (*i.e.* at 38754 or 38814). Sequence analysis allowed me to show which one of these sites was ligated during construction. In short, the 38814 site was the site used to fuse the *cro* and *O* genes together in pHB30.

Thus, it is still not known why the pHB30$^{nl\text{-}42}$ isolates #14 and #16 do not complement a *Pam3* infecting phage. The *P* genes of these two specific isolates were never actually sequenced, thus it remains likely that these two constructs contain mutations within gene *P*.

In summary, it appears that pHB30$^{nl\text{-}42}$ isolates 1-13, 15, and 17-22 do not express wild type P protein at 42°C because the *cI*[ts]857 mutation reverted to *cI* wt. Isolates 14 and 16 retain the *cI*[ts] mutation, and likely contain interesting mutations within gene *P* that suppress the inhibitory effect of P expression on host metabolism.

3.1.5. Transient P Induction Assays

The transformation assay system utilized above can be described as an "all or none" system, meaning that it is not very sensitive. If P protein were inhibiting cell metabolism/growth/development, cells would be unable to divide to form colonies on indicator plates, producing the 'lethal' phenotype as seen by Maiti *et al.* (1991a). Cell survival also requires shutting down *P* gene expression, *i.e.* requiring either the accumulation of sufficient CI repressor (made from the introduced plasmid) or some

form of spontaneous mutation that incapacitates *P* expression or activity (either plasmid or host encoded).

I wished to use a more sensitive assay for measuring the 'P-effect' on host cells. It was decided to transiently expose cells to high levels of P protein for up to five hours and measure cell growth, cell survival and cell morphology.

3.1.5.1. P-Interference

594 *E. coli* cells containing either pHB30 (P^+), pHB31 (P^-), pHB33 (P^-) or pHB35 (P^-) were grown to early log phase (A_{575nm} ~0.1-0.2) at 30°C in TB+Amp. At time 0 (A_{575nm} ~0.1-0.2), the cultures were swirled in a 50°C water bath for 15 seconds and incubated with shaking at 42°C. Every 30 minutes, absorbance readings were taken and every 60 minutes culture aliquots were removed to be used for determining cell viabilities. Cell viability was measured as the culture cell titer at time X / the cell titer of the culture at time 0.

The absorbance values taken at A_{575nm} were used as a measurement of attained culture turbidity and cell growth. As shown in Fig. 3.3A, culture turbidity / cell growth values increased over time for every strain tested (*i.e.* 594, 594[pHB30], 594[pHB31], 594[pHB33] and 594[pHB35]). Growth of cells containing plasmid pHB31 was slightly inhibited. Cells containing pHB30 (*i.e.* expressing P protein) demonstrated the greatest inhibition of cell growth.

The four plasmids demonstrated a more severe effect when cell survival was directly assayed, Fig. 3.3B and Table 3.6. All four plasmids had a negative effect on cell survival when compared to 594 alone; suggesting that maintenance of the

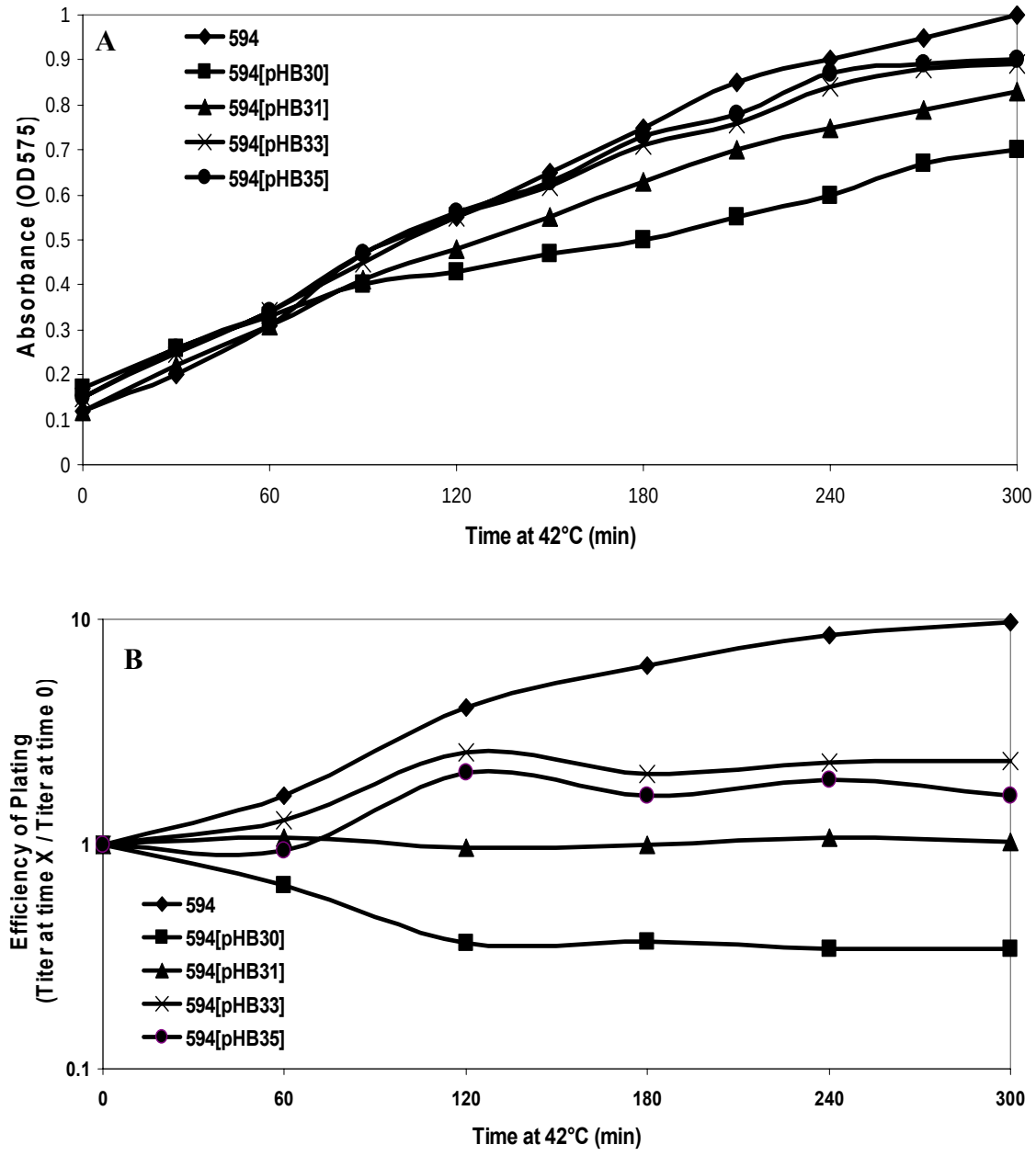

Figure 3.3. Transient induction of plasmids pHB30, pHB31, pHB33 and pHB35. Early log phase 594 cells (A_{575nm} ~ 0.1-0.2) were shifted from 30°C to 42°C for 5 hours. Absorbance readings (A_{575nm}) were taken at indicated time intervals. Culture aliquots were removed and plated on Tryptone Broth (TB) + Ampicillin (50 μg/mL) plates at 30°C to assay for cell survival. Curves represent the averages of at least two independent trials. Standard error values, not presented, were ≤ 0.1 in A and ≤ 1.86 in B.

Table 3.6. Effect of P^+ and P^- Plasmids on 594 Host Cell Viabilities Following a 5 Hour Incubation at 42°C

Host Strain	Relative Efficiency of Plating (EOP) [a] +/- Standard Error
594	9.78 +/- 1.21
594[pHB30]	0.34 +/- 0.14
594[pHB31]	1.02 +/- 0.47
594[pHB33]	2.35 +/- 0.14
594[pHB35]	1.65 +/- 0.31

[a] Early log phase cells (A_{575nm} ~ 0.15) were shifted from 30°C to 42°C for five hours; at which point a culture aliquot was removed and streaked onto a TB (TB+Amp for plasmid strains) plate and incubated overnight at 30°C. The resultant colonies were counted. Results are presented as relative efficiency of plating (EOP) = Cell titer at 5 hours at 42°C / Cell titer at 0 hours at 42°C.

plasmids was slightly detrimental to the host cells. During the five hour interval at 42°C, the number of 594 cells increased approximately 10-fold. Cells containing pHB33 or pHB35 (deleted for the C-terminal portion of gene *P*) increase in cell titer by about 2-fold. Cells containing pHB31 (containing an in-frame N-terminal deletion of gene *P*) did not increase in number, suggesting that the P protein fragment produced is detrimental to host cell development. Cells containing pHB30 decreased in titer by about 3-fold, demonstrating that the over-expression of P protein was lethal to about 65% of the cells, *i.e.* a viability of 0.34. Alternatively, cells having lost pHB30 would also not be able to form colonies on TB+Amp plates, thus it can be said that 35% of cells expressing *P* for five hours survived to form Amp^R colonies; 65% of cells are either killed due to the lethal effects of *P* expression or have lost the *P* expressing plasmid.

Gram staining of the cells used to generate Fig. 3.3 explained the seeming discrepancy between absorbance values and cell survival data. A_{575nm} of 594[pHB30] cells grown at 42°C continued to increase even though cell titer was decreasing. Gram staining revealed that the cells exposed to P protein expressed from pHB30 became filamented. This filamentation was specific to pHB30 as 594 and 594[pHB31] cells were also assayed and no filamented cells were seen. 594[pHB30] cells grown at 42°C for five hours appeared 2-3 X longer than 594 cells or 594[pHB31] cells grown at 42°C (data not shown).

Because 35% of the initial cell population that were transiently exposed to P protein survived to form colonies, the term P-killing or P-lethality was re-named P-Interference of cell metabolism, *i.e.* P-Interference.

3.1.5.2. P-Interference Host Strain Variation

Several strains of *E. coli* were assayed in order to see if susceptibility to P-Interference varied among wild type host strains, Fig. 3.4 and Table 3.7. SA500, which was previously believed to be an *E. coli* strain naturally resistant to P-killing (Hayes, unpublished data), was shown to be sensitive to P-Interference, even more so than 594. W3350 was also shown to be susceptible to P-Interference. While W3350 cell growth (A_{575nm}) was greatly inhibited by P over-expression, Fig. 3.4A, the P-mediated decrease in Amp^R cell titer was similar to 594, Fig. 3.4B, Table 3.7 . In summary, the three common *Eco*K laboratory strains of *E. coli* assayed were found to be nearly identical in their susceptibility to P-Interference as expressed from the plasmid pHB30 at 42°C.

3.1.5.3. Killing *in cis* vs. Killing *in trans*

P protein, produced in a cell from the plasmid pHB30, is a product capable of acting *in trans* to complement for a defective P protein made by an infecting λ*imm*434*cI⁻Pam3* phage. As such, it can be assumed that the killing activity associated with over-expressed P protein, is due to its ability to diffuse through the cell and act *in trans*.

In contrast, Y836 cells, containing a cryptic λ*cI*857 prophage fragment undergo not only *O* and *P* gene expression, but their products trigger λ replication initiation from *ori*λ. When cells with a cryptic prophage are shifted to 42°C, λ replication initiation is induced, thus undergoing a process called replicative killing or killing *in cis* (Hayes *et al,* 1983; Hayes and Hayes, 1986;Hayes *et al*, 1998). The λ

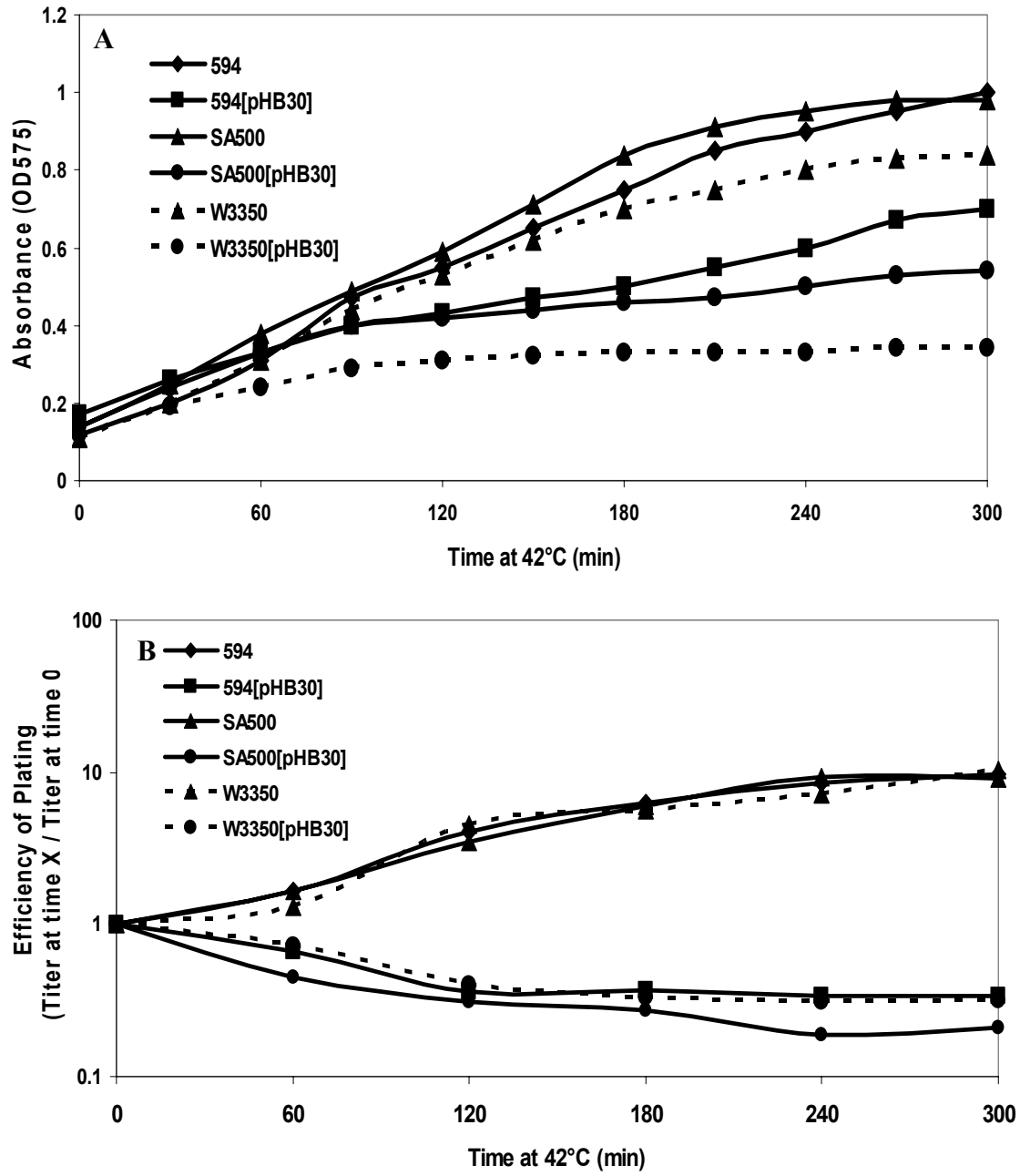

Figure 3.4. Variation in Host Strain Susceptibility to P-Interference. Early log phase cells (A_{575nm} ~ 0.1-0.2) were shifted from 30°C to 42°C for 5 hours. Absorbance readings (A_{575nm}) were taken at indicated time intervals. Culture aliquots were removed and plated on Tryptone Broth (TB) + Ampicillin (50µg/mL) plates at 30°C to assay for cell survival. Curves represent the averages of at least two independent trials. Standard error values, not presented, were ≤ 0.1 in A and ≤ 1.86 in B.

Table 3.7. Host Strain Variation in Susceptibility to P-Interference

Host Strain	Relative Efficiency of Plating [a] +/- Standard Error
594	9.78 +/- 1.21
594[pHB30]	0.34 +/- 0.14
SA500	9.17 +/- 0.30
SA500[pHB30]	0.21 +/- 0.01
W3350	10.27 +/- 0.32
W3350[pHB30]	0.32 +/- 0.12

[a] Early log phase cells (A_{575nm} ~ 0.15) were shifted from 30°C to 42°C for five hours; at which point a culture aliquot was removed and streaked onto a TB (TB+Amp for plasmid strains) plate and incubated overnight at 30°C. The resultant colonies were counted. Results are presented as Relative efficiency of plating (EOP) = Cell titer at 5 hours at 42°C / Cell titer at 0 hours at 42°C.

genome begins to replicate upon the de-repression of transcription (*cI*[ts]857 is inactive at 42°C) from a cryptic prophage that remains integrated within the host chromosome. Propagating λ replication forks arising from *ori*λ spread into the adjacent host chromosome and may collide with *E. coli* replication forks.

In order to contrast P-Interference, shown in Fig. 3.3 and 3.4 (which we have also dubbed killing *in trans*), with killing *in cis*, Y836 cells were grown up at 30°C, were then shifted to 42°C to thermally induce the cryptic prophage, and incubated at 42°C for 5 hours. In a parallel culture, the plasmid pHB30 was also transformed into the Y836 cells in an attempt to assess the cumulative effects of both *cis* and *trans* killing, Fig. 3.5 and Table 3.8. Y836 cells were killed effectively at 42°C (*cis* killing). When Y836[pHB30] cells were shifted to 42°C, both *cis* and *trans* killing were expected to occur within the host cells. In actuality, the over-expression of *P* from pHB30 reduced the level of killing seen in Y836. The simplest interpretation is that the over-expression of *P* from pHB30 interferes with λ replication initiation.

As ongoing research in the Hayes laboratory is focused on furthering the understanding of replicative killing, I decided to study P-Interference in greater detail.

3.1.5.4. P-Interference is Rapidly Reversible

A simple experiment was done in order to determine how rapidly cells are able to recover after *P* gene expression is turned off. *P* gene expression in pHB30 is regulated by a temperature sensitive CI repressor protein. When cells are grown at 42°C, the repressor is inactivated and high levels of P protein are made. When the cells are shifted back to 30°C, the repressor protein is rapidly renatured (Slavcev and

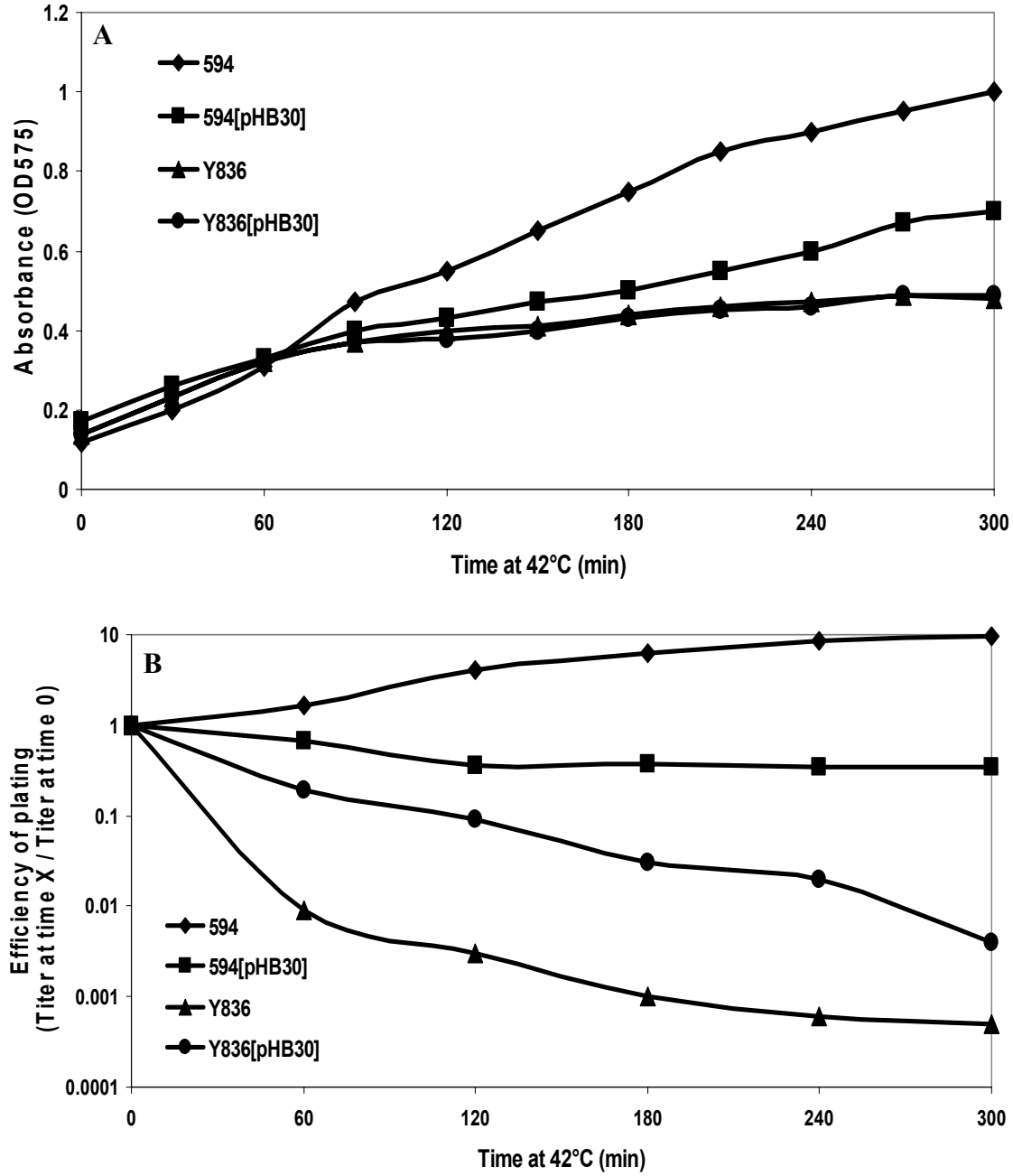

Figure 3.5. Killing *in cis* and *in trans*. Early log phase cells (A_{575nm} ~ 0.1-0.2) were shifted to 42°C for 5 hours. Absorbance readings (A_{575nm}) were taken at indicated time intervals. Culture aliquots were removed and plated on Tryptone Broth (TB) + Ampicillin (50 μg/mL) plates at 30°C to assay for cell survival. Curves represent the averages of at least two independent trials. Standard error values, not presented, were ≤ 0.1 in A and ≤ 1.86 in B.

Table 3.8. Killing *in cis* and *in trans* in Host Cells Incubated at 42°C for 5 Hours

Host Strain	Relative Efficiency of Plating (EOP) [a] +/- Standard Error
594	9.78 +/- 1.21
594[pHB30]	0.34 +/- 0.14
Y836	0.0005 +/- < 0.00001
Y836[pHB30]	0.004 +/- 0.001

[a] Early log phase cells (A_{575nm} ~ 0.15) were shifted from 30°C to 42°C for five hours; at which point a culture aliquot was removed and streaked onto a TB (TB+Amp for plasmid strains) plate and incubated overnight at 30°C. The resultant colonies were counted. Results are presented as Relative efficiency of plating (EOP) = Cell titer at 5 hours at 42°C / Cell titer at 0 hours at 42°C.

Hayes, 2005a), in turn blocking *P* gene expression.

Two parallel 594[pHB30] cultures were grown at 42°C for 4 hours, until the A_{575nm} curves began to level off. At this point, one culture was retained at 42°C, while the other was placed at 30°C. Absorbance levels of the two cultures were monitored for one more hour. Within 20 minutes of the down shift from 42°C to 30°C (*i.e.* turning off *P* gene expression) the culture absorbance values began to dramatically increase, Fig. 3.6. Simultaneously, by 20 minutes after the shift down from 42°C to 30°C, the proportion of filamented cells also visually decreased (data not shown). These results suggested that the host cell contains a fast mechanism for removing excess P protein.

3.1.6. Influence of Host Mutations on P-Interference

The results from the repressor renaturation experiment suggested that the host cell contains a mechanism for degrading or removing P protein from the cell. We expected that P-Interference would be enhanced in cells made defective in activities that normally served to remove or metabolize induced P activity as expressed from pHB30. Several 594 derivatives were assayed for P-Interference in order to further understanding of P action and metabolism within the host cell.

3.1.6.1. Host Cells Defective for the ClpXP Protease are Highly Sensitive to P-Interference

The hypothesis that λ P may exist in a partially denatured state in the cell, making it more susceptible to host heat shock proteins, chaperones and proteases

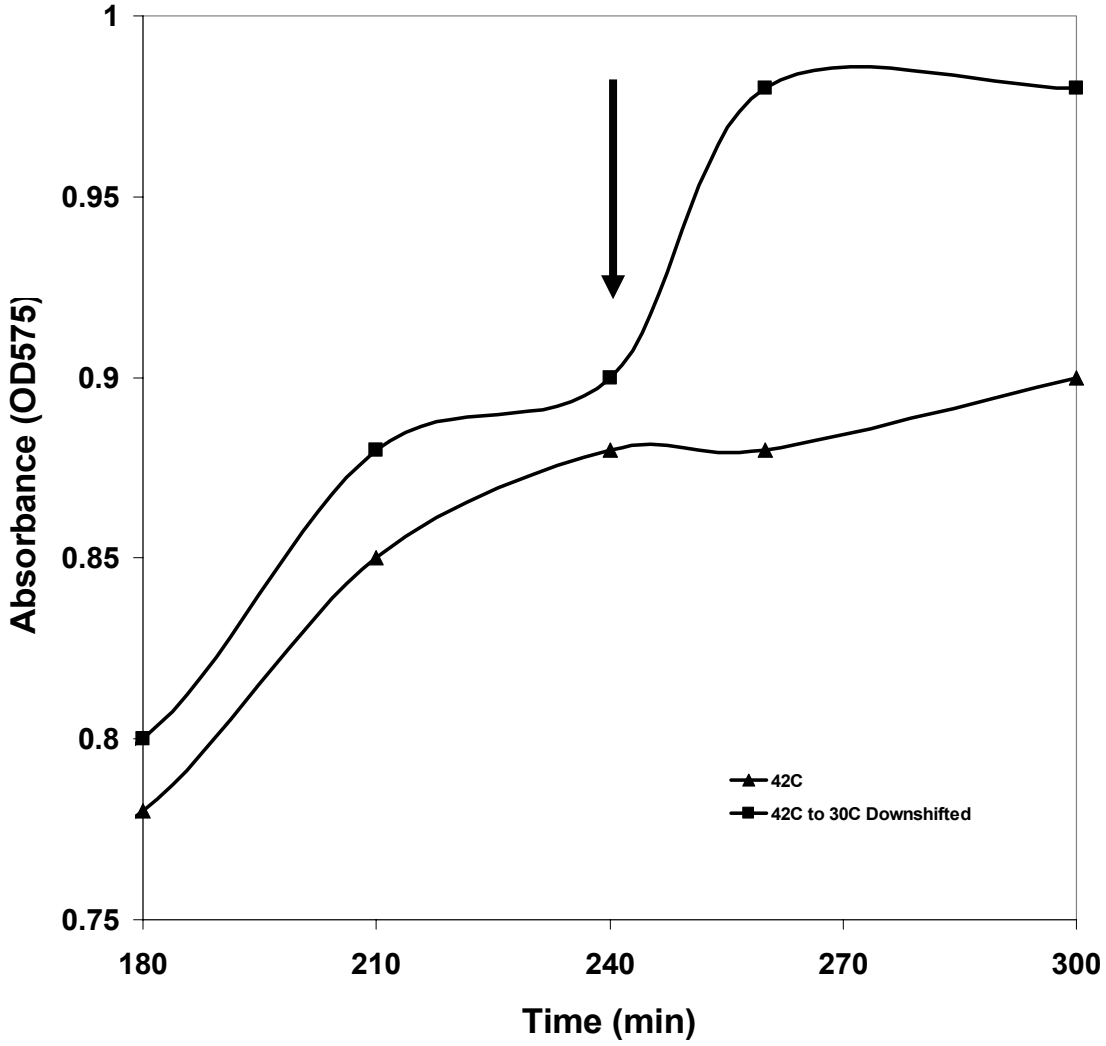

Figure 3.6. P-Interference is Rapidly Reversible. Two parallel cultures of 594[pHB30] were grown at 42°C until the A_{575nm} values began to level off (*i.e.* at 240 min; the black arrow). At this time, one culture was left at 42°C. The second culture was downshifted to 30°C. Absorbance readings were taken at the indicated intervals.

(Hoffmann *et al,* 1992), led me to explore the possibility that a specific cellular protease targets P for degradation. However, it remains unknown if the natural state of P is denatured or properly folded.

Protease defective 594 derivative host strains were tested for sensitivities to P-Interference using the transient P induction assay described in section 3.1.5.2. Among several protease mutants, I found that cells containing a ClpXP defect (the 594 *clpP*::kan insertion also inactivates the downstream *clpX* gene; Gottesman *et al*, 1998) were made more sensitive to P-Interference, Fig. 3.7 and Table 3.9; cell growth was inhibited compared to 594 cells, Fig. 3.7A, and cell viabilities were about 50-fold lower, Fig. 3.7B and Table 3.9. This result suggested that the ClpXP protease is responsible for the rapid recovery from *P* expression. The double mutant host strain 594 *clpP*::kan Δ*lon* was less susceptible to P-Interference than the ClpXP mutant by itself. Somehow, the Lon defect appears to partially suppress the ClpXP defect. Cells containing a defect in ClpB or HflA both showed much better cell growth than 594 cells, Fig. 3.7A; however, while cell viabilities for ClpB defective cells were slightly inhibited, HflA defective cells showed viabilities similar to 594, Fig. 3.7A and Table 3.9. Cells containing a defect in ClpA or SsrA both showed an inhibition of cell growth, Fig. 3.7A, while maintaining cell viabilities comparable to 594 cells, Fig. 3.7B and Table 3.9. Although the growth curves for the assorted protease defective 594 derivatives showed various levels of inhibition; only a defect in ClpXP severely affected cell viabilities.

3.1.6.2. Influence of *lexA*3(Ind$^-$) Mutation on P-Interference

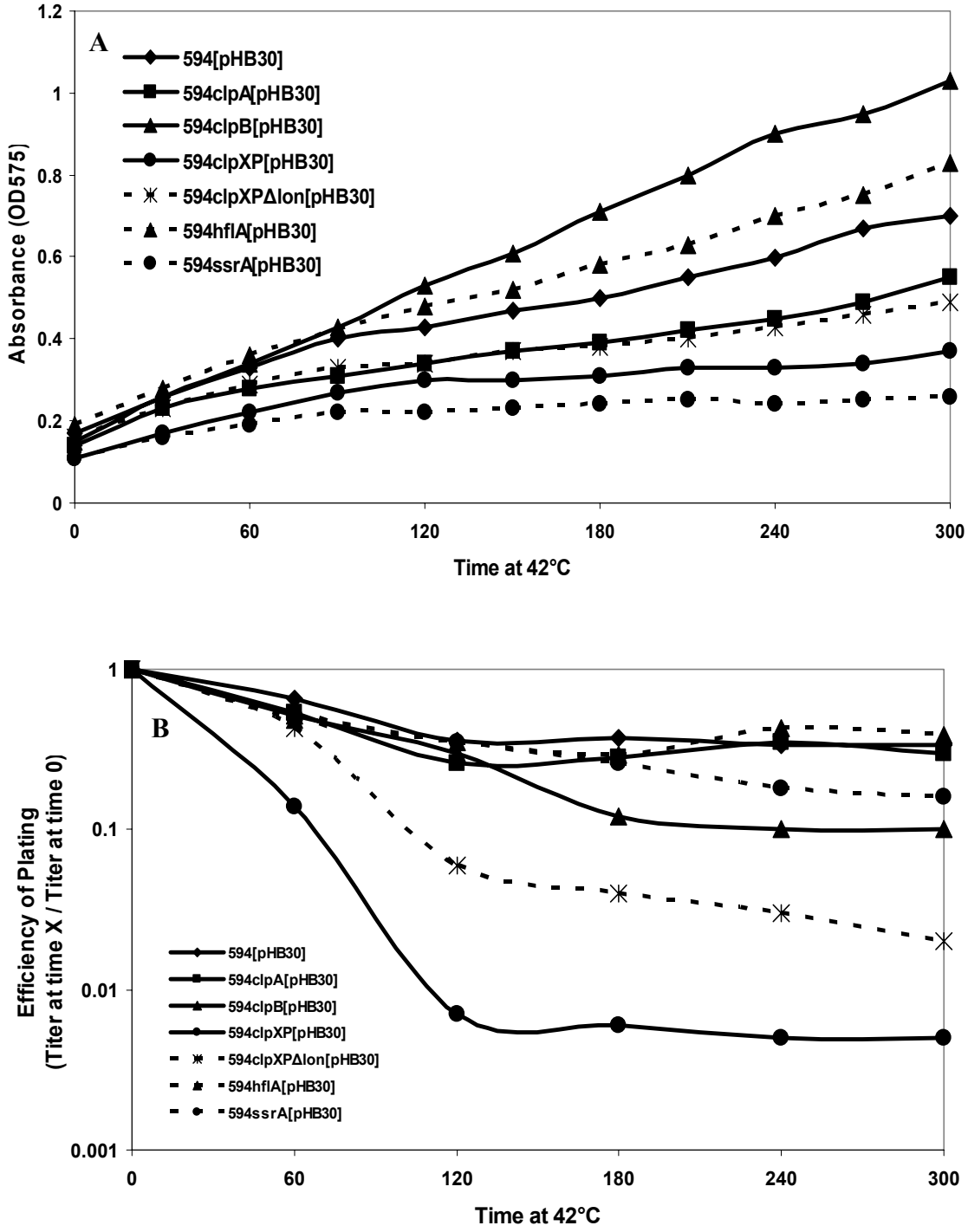

Figure 3.7. Effect of Protease Mutations on P-Interference. Early log phase cells ($_{A575nm}$ ~ 0.1-0.2) were shifted to 42°C for 5 hours. Absorbance readings (A_{575nm}) were taken at indicated time intervals. Culture aliquots were removed and plated on Tryptone Broth (TB) + Ampicillin (50 μg/mL) plates at 30°C to assay for cell survival. Curves represent the averages of at least two independent trials. Standard error values, not presented, were ≤ 0.15 in A and ≤ 0.33 in B.

Table 3.9. Effect of Host Protease Mutations on P-Interference

Host Strain	Relative Efficiency of Plating (EOP) [a] +/- Standard Error
594[pHB30]	0.34 +/- 0.14
594 *clpA*::kan [pHB30]	0.30 +/- 0.04
594 *clpB*::kan [pHB30]	0.10 +/- 0.01
594 *clpP*::kan [pHB30]	0.005 +/- 0.005
594 *clpP*::kan Δ*lon* [pHB30]	0.02 +/- 0.01
594 *hflA*::kan [pHB30]	0.39 +/- 0.06
594 *ssrA*::cat [pHB30]	0.16 +/- 0.01

[a] Early log phase cells (A_{575nm} ~ 0.15) were shifted from 30°C to 42°C for five hours; at which point a culture aliquot was removed and streaked onto a TB (TB+Amp for plasmid strains) plate and incubated overnight at 30°C. The resultant colonies were counted. Results are presented as Relative efficiency of plating (EOP) = Cell titer at 5 hours at 42°C / Cell titer at 0 hours at 42°C.

The absorbance of cell cultures exposed to P protein expressed from pHB30 continued to increase even though viable cell titers were decreasing, Fig. 3.3. This effect was shown to be due to P- mediated cellular filamentation. Even though cell numbers were dropping, the length of each surviving cell was increasing, leading to a net increase in absorbance at 575nm.

It was essential to determine if P was directly responsible for the cellular filamentation phenotype, or if it was playing an indirect role (*i.e.* induction of SOS-mediated cellular filamentation). In order to examine the possibility that the SOS response was involved in the filamentation seen upon P over-expression, 594*lexA*3 *malF*3089::Tn*10* cells, with or without pHB30, were grown at 42°C for 5 hours. At the indicated time intervals, absorbance and efficiency of plating values were measured, Fig. 3.8 and Table 3.10.

The *lexA3* mutation, which prevents induction of the SOS response, inhibited the absorbance increase seen upon P over-expression, Fig. 3.8A. Gram stains (data not shown) revealed that cells defective for SOS induction did not undergo filamentation upon exposure to P from the plasmid pHB30. This result suggested that P-mediated cell filamentation was caused by the induction of the host's SOS response. Cells defective for SOS induction also exhibited a small but reproducible enhancement in P-mediated cell killing, Fig. 3.8B and Table 3.10. These results suggested that the SOS response plays a role in protecting the cell from high levels of P protein.

3.1.6.3. Influence of *dnaB* Mutations on P-Interference

Two *E. coli* strains, GrpD55 and GrpA80, previously shown to be resistant to

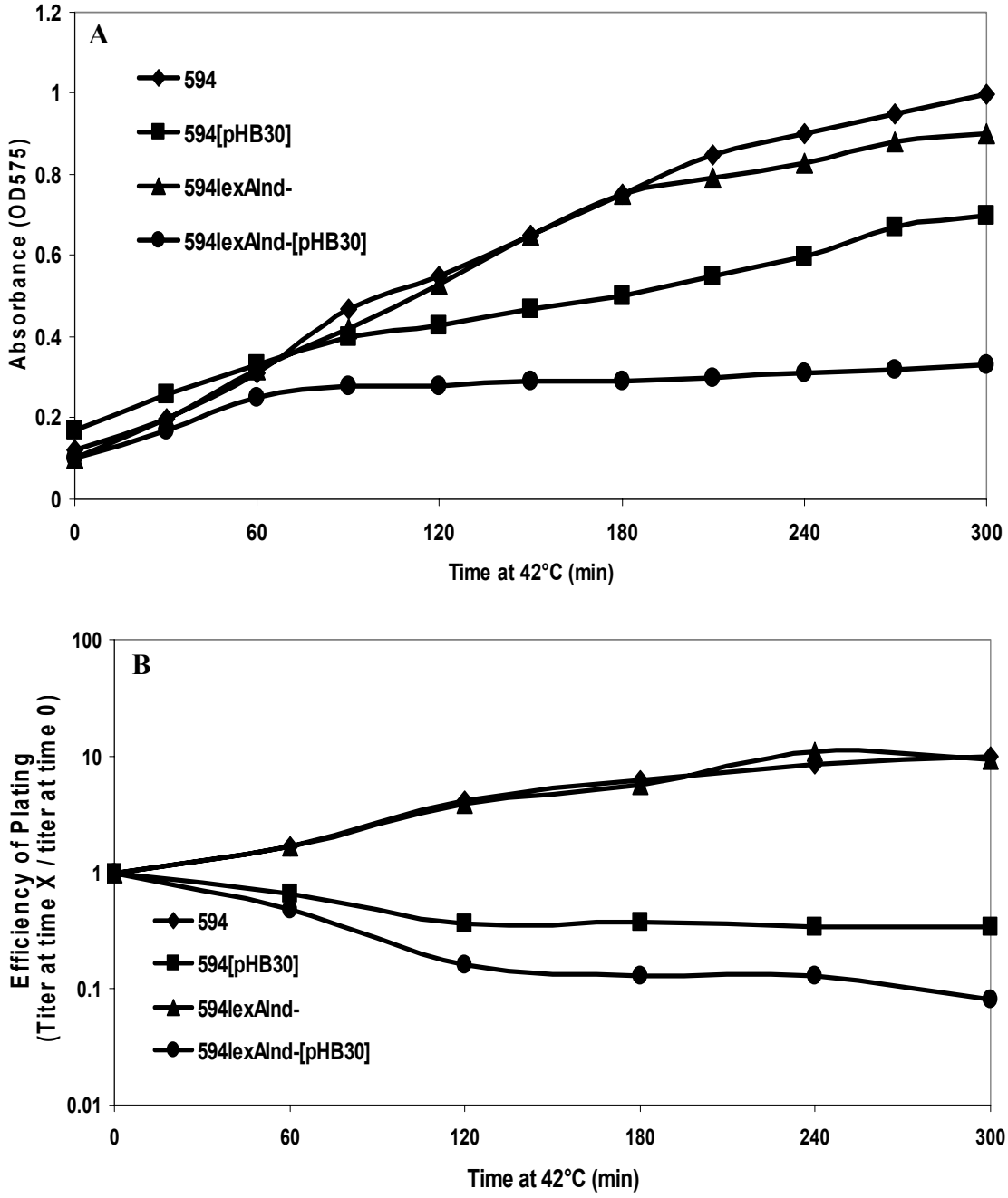

Figure 3.8. Effect of an SOS Mutation on P-Interference. Early log phase cells (A_{575nm} ~ 0.1-0.2) were shifted to 42°C for 5 hours. Absorbance readings (A_{575nm}) were taken at indicated time intervals. Culture aliquots were removed and plated on Tryptone Broth (TB) + Ampicillin (50 μg/mL) plates at 30°C to assay for cell survival. Note the designation 594lexAInd- refers to strain 594 *lexA*3(Ind$^-$). Curves represent the averages of at least two independent trials. Standard error values, not presented, were ≤ 0.1 in A and ≤ 2.81 in B.

Table 3.10. Effect of a *lexA3* Mutation on P-Interference

Host Strain	Relative Efficiency of Plating (EOP) [a] +/- Standard Error
594	9.78 +/- 1.21
594[pHB30]	0.34 +/- 0.14
594 *lexA*3	9.19 +/- 2.67
594 *lexA*3 [pHB30]	0.08 +/- 0.03

[a] Early log phase cells (A_{575nm} ~ 0.15) were shifted from 30°C to 42°C for five hours; at which point a culture aliquot was removed and streaked onto a TB (TB+Amp for plasmid strains) plate and incubated overnight at 30°C. The resultant colonies were counted. Data is presented as Relative efficiency of plating (EOP) = Cell titer at 5 hours at 42°C / Cell titer at 0 hours at 42°C.

P-killing in a transformation assay similar to the one used by Maiti *et al.* in 1991 (Bull, 1995), were believed to contain mutations within their *dnaB* genes. The defect in the GrpA80 strain was mapped to *dnaB* by Saito and Uchida (1977), while the defect in GrpD55 was mapped to *dnaB* by Bull and Hayes (1996).

3.1.6.3.1. Influence of the *grpD*55 Mutation on P-Interference

594 *grpD*55 cells were grown at 42°C for 5 hours. Absorbance and plating efficiency readings were taken at the indicated time intervals, Fig. 3.9 and Table 3.11. The *grpA*80 defect was not tested, as the allele could not be transduced from the original strain received from Dr. Uchida into the isogenic host 594.

As shown in Fig. 3.9, the addition of the *grpD*55 allele to 594 had no discernible influence on P-Interference. The 594 *grpD*55 and 594 *grpD*55[pHB30] strains appear to grow more quickly than the 594 derivatives, as seen by higher A_{575nm} values, Fig. 3.9A. However, the cell survival curves for 594 and 594*grpD*55 derivatives are almost identical, Fig. 3.9B and Table 3.11.

The double mutant 594 *clpP::kan grpD*55 was also tested for susceptibility to P-Interference, Fig. 3.10 and Table 3.11. The presence of the *grpD*55 allele appears to suppress the extreme susceptibility of the ClpXP deficient cells.

The introduction of *grpD*55 into Y836 prevents *cis* killing at 42°C (Hayes *et al,* 2005). The susceptibility of Y836 *grpD*55 cells to P-Interference was investigated, and the results are illustrated in Fig. 3.11 and Table 3.11. As can be seen from Fig. 3.11, the presence of the *grpD*55 allele made Y836 cells immune to P-Interference.

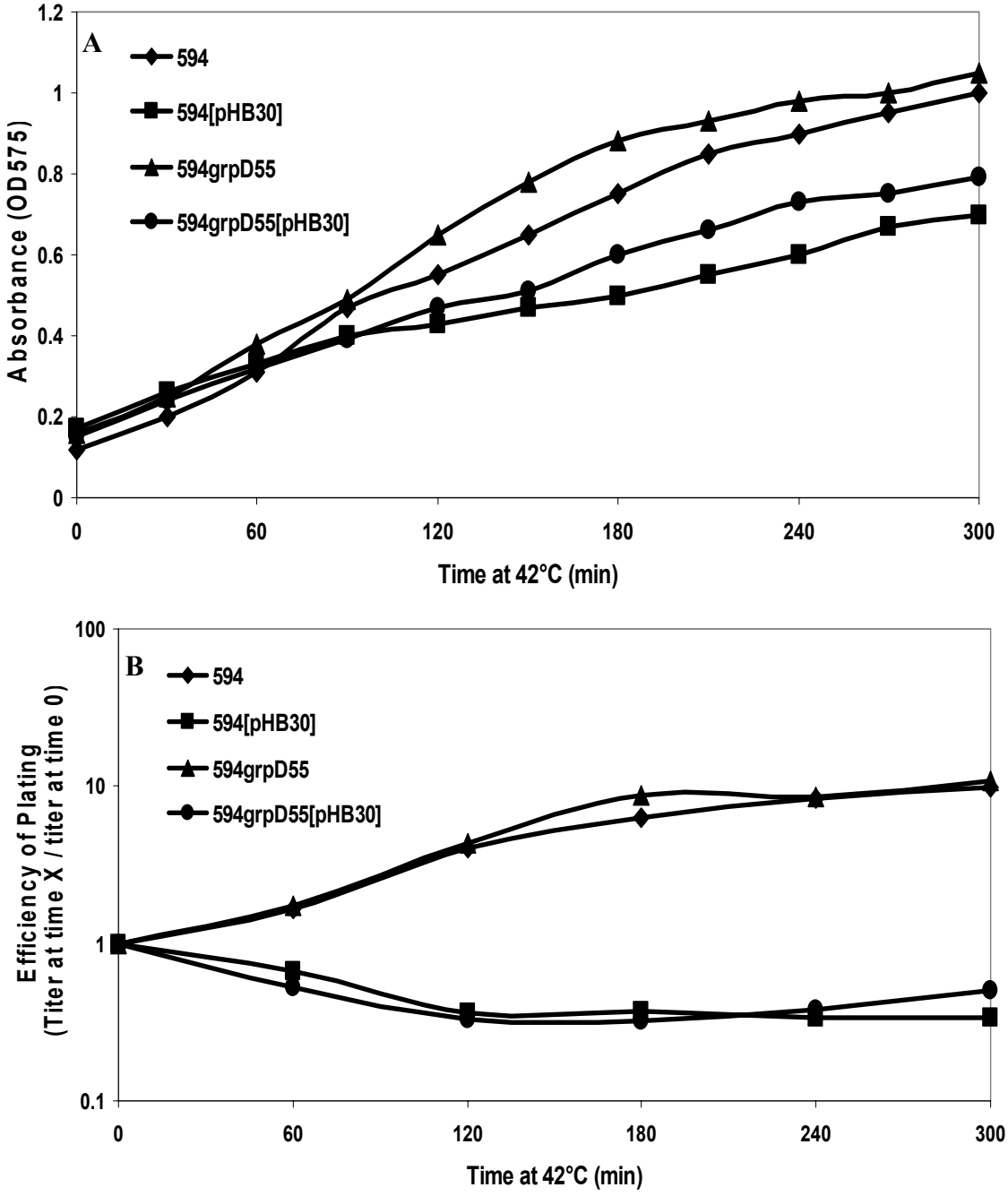

Figure 3.9. Influence of the *grpD*55 Mutation on P-Interference. Early log phase cells (A_{575nm} ~ 0.1-0.2) were shifted to 42°C for 5 hours. Absorbance readings (A_{575nm}) were taken at the indicated time intervals. Culture aliquots were removed and plated on Tryptone Broth (TB) + Ampicillin (50 μg/mL) plates at 30°C to assay for cell survival. Curves represent the averages of at least two independent trials. Standard error values, not presented, were ≤ 0.1 in A and ≤ 1.86 in B.

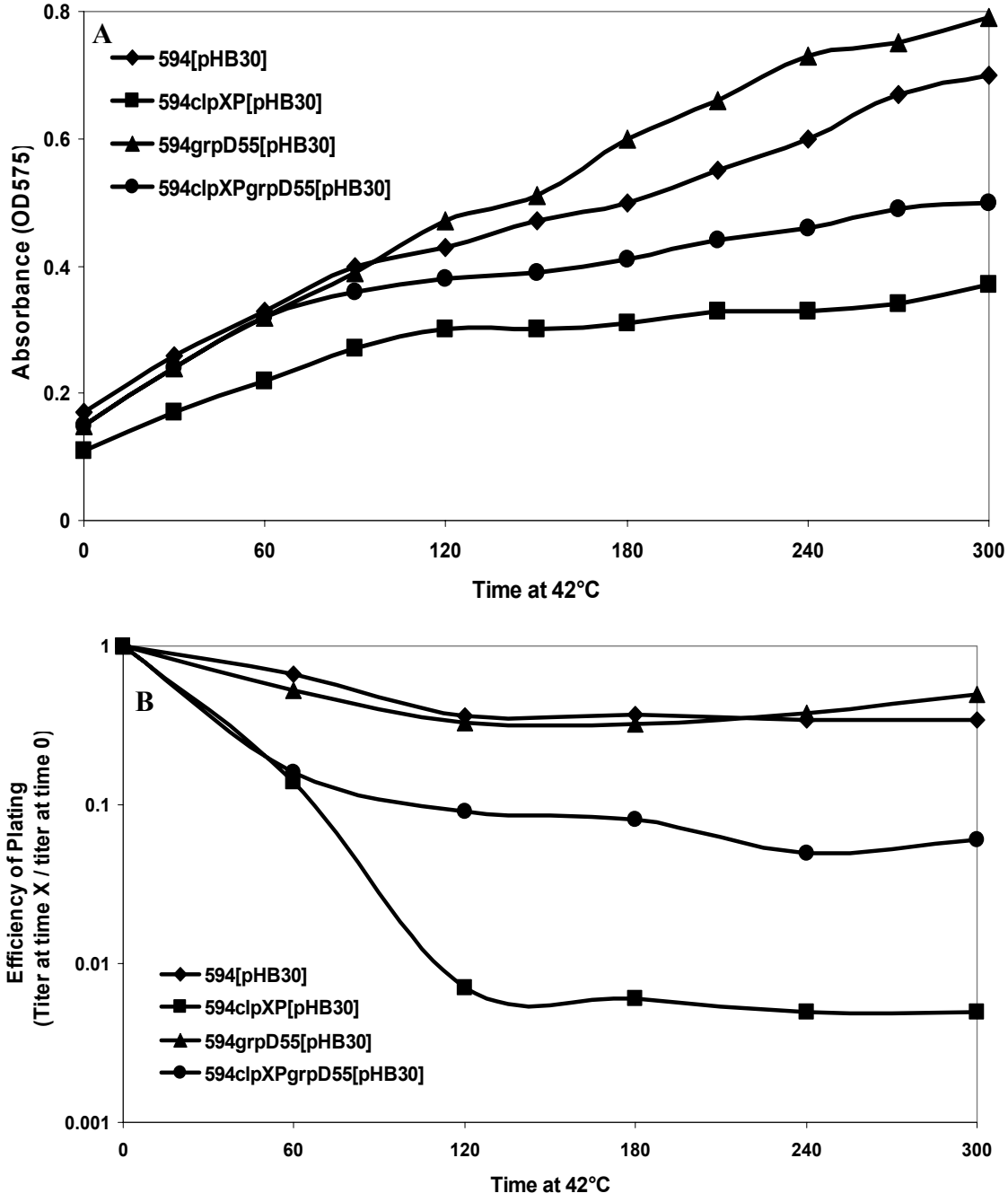

Figure 3.10. Influence of Simultaneous *clpP*::kan and *grpD*55 Mutations on P-Interference. Early log phase cells (A_{575nm} ~ 0.1-0.2) were shifted to 42°C for 5 hours. Absorbance readings (A_{575nm}) were taken at indicated time intervals. Culture aliquots were removed and plated on Tryptone Broth (TB) + Ampicillin (50 μg/mL) plates at 30°C to assay for cell survival. Curves represent the averages of at least two independent trials. Standard error values, not presented, were ≤ 0.14 in A and ≤ 2.61 in B.

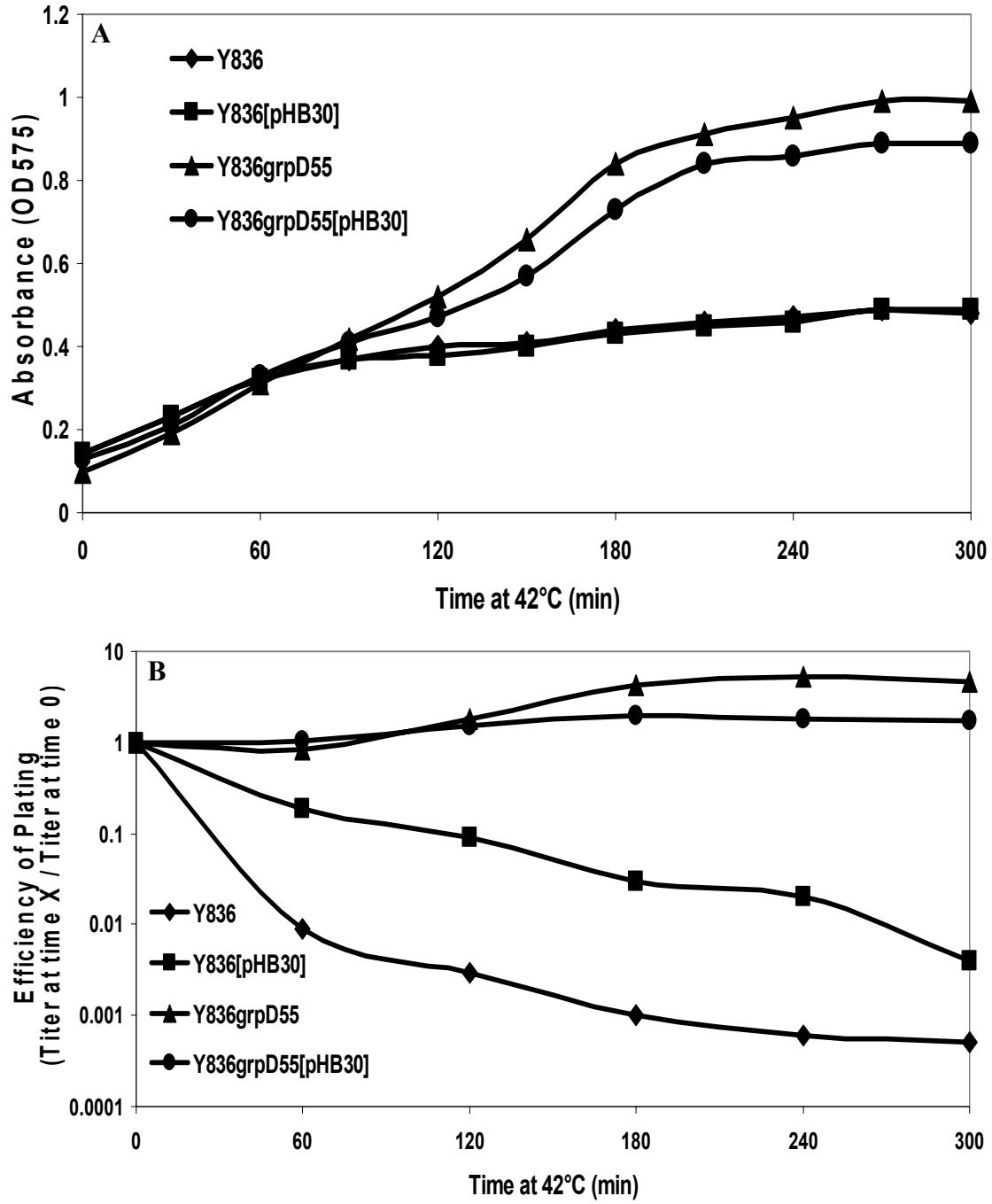

Figure 3.11. Influence of the *grpD*55 Mutation on *cis* and *trans* Killing from Y836. Early log phase cells (A_{575nm} ~ 0.1-0.2) were shifted to 42°C for 5 hours. Absorbance readings (A_{575nm}) were taken at indicated time intervals. Culture aliquots were removed and plated on Tryptone Broth (TB) + Ampicillin (50 μg/mL) plates at 30°C to assay for cell survival. Curves represent the averages of at least two independent trials. Standard error values, not presented, were ≤ 0.04 in A and ≤ 0.96 in B.

Table 3.11. Effect of the *grpD*55 Mutation on *cis* and *trans* Killing

Host Strain	Relative Efficiency of Plating (EOP) [a] +/- Standard Error
594	9.78 +/- 1.21
594[pHB30]	0.34 +/- 0.14
594 *grpD*55	10.76 +/- 0.64
594 *grpD*55 [pHB30]	0.5 +/- 0.06
594 *clpP*::kan [pHB30]	0.005 +/- 0.005
594 *clpP*::kan *grpD*55 [b]	11.71 +/- 1.9
594 *clpP*::kan *grpD*55 [pHB30]	0.06 +/- 0.03
Y836	0.0005 +/- < 0.00001
Y836[pHB30]	0.004 +/- 0.001
Y836 *grpD*55	4.55 +/- 0.04
Y836 *grpD*55 [pHB30]	1.74 +/- 0.96

[a] Early log phase cells (A_{575nm} ~ 0.15) were shifted from 30°C to 42°C for five hours; at which point a culture aliquot was removed and streaked onto a TB (TB+Amp for plasmid strains) plate and incubated overnight at 30°C. The resultant colonies were counted. Results are presented as Relative efficiency of plating (EOP) = Cell titer at 5 hours at 42°C / Cell titer at 0 hours at 42°C.

[b] The strain 594 *clpP*::kan *grpD*55 was constructed by transducing the *clpP*::kan allele into 594*grpD*55 cells via phage P1. Successful transductants were selected by resistance to kanamycin and tetracycline and the ability to inhibit λ*cI*857 development while allowing the development of λ*cI*857(*18,12*)P22.

3.1.6.3.2. Sequencing Results of GrpD55 and GrpA80 Host Strains

The GrpD55 and GrpA80 strains, received from Dr. Uchida, were originally isolated as host strains unable to support λ replication. The mutations have never been localized via DNA sequencing. Saito and Uchida (1977) showed that the GrpA80 defect co-transduced with *malB* at approximately 91.5 minutes, similar to the *dnaB* locus. Saito and Uchida (1977) showed that the GrpD55 defect co-transduced with *aroE* at 71.5 minutes, and suggested that *grpD* was a new locus, essential for λ replication initiation. Further study demonstrated that the *grpD*55 allele had been assigned to an incorrect map position. In 1996, Bull and Hayes showed that the *grpD*55 allele actually co-transduced with *malF*3089::Tn*10* at 91.5 minutes, and a *dnaB*-expressing plasmid was able to fully complement for the GrpD55 defect in λ replication initiation (Bull and Hayes, 1996), strongly suggesting that *grpD*55 was an allele of *dnaB*.

The primers DnaB-1 and DnaB-6, which flank the coding sequence for the *dnaB* gene in *E. coli* K12 (NCBI Genbank accession number U00096), were used to amplify the *dnaB* genes of the strains GrpA80 and GrpD55, both received from Dr. Uchida. The resultant PCR fragments were sent to NRC-PBI for sequencing along with 3 overlapping primer pairs (*i.e.* DnaB-1 and DnaB-2; DnaB-3 and DnaB-4; DnaB-5 and DnaB-6) in order to sequence the entire gene on both strands of DNA from approximately 4262244-4263813 on the *E. coli* K12 genome (*i.e.* approximately 1570 bp was sequenced, encompassing the entire *dnaB* coding sequence).

Each *E. coli* strain, GrpA80 and GrpD55, contained two missense mutations within each of their *dnaB* genes, as shown Table 3.12. Both strains contained one

Table 3.12. Sequencing Results for the *dnaB* Genes of *E. coli* K12 Strains GrpD55 and GrpA80

Strain	Nucleotide position (*E. coli* K12) and mutation seen [a]		Amino acid substitution seen	GenBank Accession Number
GrpD55	4,263,102	G to A	V256I	DQ324465
	4,263,612	G to A	E426K	
GrpA80	4,263,349	G to A	G338E	DQ324464
	4,263,612	G to A	E426K	

[a] The *dnaB* genes were PCR amplified with primers DnaB-1 and DnaB-6. The PCR fragments were sequenced at NRC-PBI with overlapping primer pairs DnaB-1 and DnaB-2; DnaB-3 and DnaB-4; DnaB-5 and DnaB-6.

mutation in common (E426K) and one mutation unique to each strain (*i.e.* V256I for GrpD55 and G338E for GrpA80). It remains to be determined if both point mutations are necessary for their conditional effect on λ replication initiation.

Now that I have obtained sequencing data to definitively demonstrate that the *grpD*55 mutation is actually an allele of *dnaB*, I will refer to the strain 594 *grpD*55 as 594 *dnaB*grpD55 until the sequencing data has been sent to GenBank and a "true" *dnaB* allele number is received.

3.1.6.4. Rpl8 (Repress P-lethality) Strain

The *E. coli* K12 strain Rpl8 was obtained from Dr. N.C. Mandal. This strain was reported to be resistant to P-killing (Datta *et al*, 2005a), as determined by a transformation assay similar to the one utilized in section 3.1.1. 594 host cells were mutagenized and then transformed with the plasmid pMR45, which constitutively expresses P protein at a high level (Maiti *et al,* 1991a; Datta *et al,* 2005a). Host cells surviving transformation (termed Rpl for **R**epress **P-l**ethality) were shown to contain point mutations within the *dnaA* gene. The Rpl mutants were also shown to be slow growing (Datta *et al*, 2005a). The strain Rpl8 was reported to contain a missense mutation in *dnaA* (N313T). Rpl8 single colony 4 was streaked out and utilized in further assays.

3.1.6.4.1. Influence of Rpl8(4) Defect on P-Interference

The Rpl8 strain was tested for its susceptibility to P-Interference, using the transient induction assay methodology, Fig. 3.12 and Table 3.13. It was seen that,

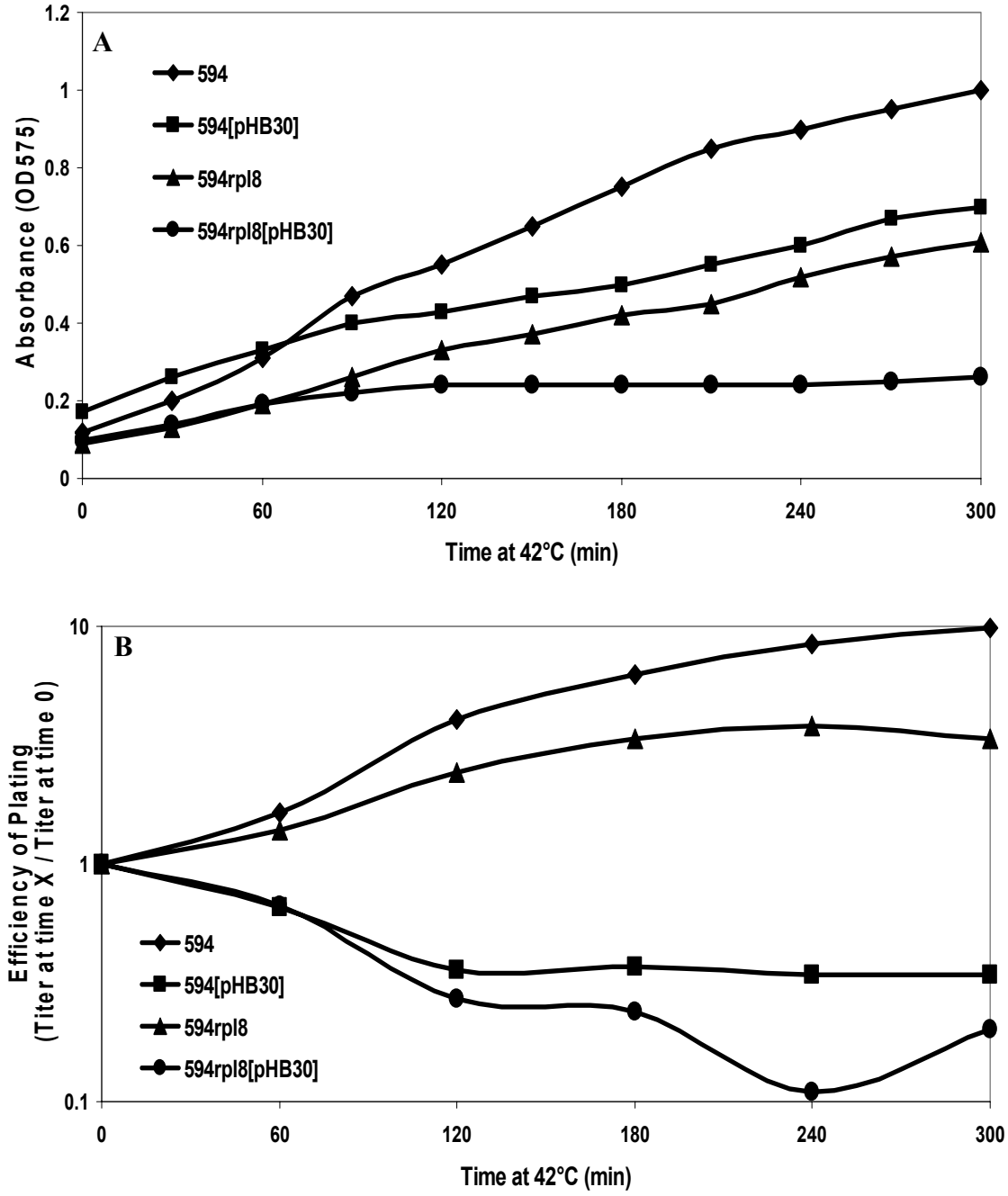

Figure 3.12. Influence of the Rpl8 Defect on P-Interference. Early log phase cells (A_{575nm} ~ 0.1-0.2) were shifted to 42°C for 5 hours. Absorbance readings (A_{575nm}) were taken at indicated time intervals. Culture aliquots were removed and plated on Tryptone Broth (TB) + Ampicillin (50μg/mL) plates at 30°C to assay for cell survival. Note that the designation 594rpl8 refers to host strain 594 *dnaA*rpl8. Curves represent the averages of at least two independent trials. Standard error values, not presented, were ≤ 0.1 in A and ≤ 1.86 in B.

Table 3.13. Effect of the *rpl*8 Mutation on P-Interference

Host Strain	Relative Efficiency of Plating (EOP) [a] +/- Standard Error
594	9.78 +/- 1.21
594[pHB30]	0.34 +/- 0.14
594 *dnaA*rpl8	3.39 +/- 0.78
594 *dnaA*rpl8[pHB30]	0.2 +/- 0.05

[a] Early log phase cells (A_{575nm} ~ 0.15) were shifted from 30°C to 42°C for five hours; at which point a culture aliquot was removed and streaked onto a TB (TB+Amp for plasmid strains) plate and incubated overnight at 30°C. The resultant colonies were counted. Results are presented as Relative efficiency of plating (EOP) = Cell titer at 5 hours at 42°C / Cell titer at 0 hours at 42°C.

although the strain was slow growing as reported by Dr. Mandal (Fig. 3.12A), it remained sensitive to P-Interference from the plasmid pHB30 at 42°C (Fig. 3.12B and Table 3.13).

3.1.6.4.2. Sequencing Results of the *dnaA* Genes of 594 and Rpl8(4)

The primers DnaA-1 and DnaA-6, which flank the coding sequence for the *dnaA* gene in *E. coli* K12, were used to amplify the *dnaA* genes of the strains 594 and Rpl8 single colony 4. The resultant PCR fragments were sent to NRC-NPBI for sequencing along with the primer pair DnaA-3 and DnaA-4. Both strands of *dnaA,* from the area reported (Datta *et al,* 2005a) to contain three separate rpl mutations, were sequenced (*i.e.* from 3880756 – 3881264). The three Rpl mutatnts were each reported to contain one missense mutation in *dnaA*; Rpl8 (N313) at 3880815, Rpl12 (Y200N) at 3881153 and Rpl14 (S246T) at 3881117. Of the three *dnaA* mutations, only Rpl8 is located on the *E. coli* chromosome; Rpl12 and Rpl14 are localized on plasmids (Datta *et al,* 2005a). Note: No *dnaA* allele numbers exist for these mutations as they have not been submitted to Genbank.

Sequence analysis demonstrated that 594 and Rpl8(4) contained a DNA sequence identical to the wild type *E. coli* K12 *dnaA* sequence over bases 3880739 - 3881280, indicating that the Rpl8(4) strain received from Dr. N.C. Mandal (and used in the P-Interference experiment described above) does not contain the reported *dnaA* mutation. Thus, I have not been able to reproduce or refute their published results reporting that P-killing can be abolished by a mutation within *dnaA*.

3.1.6.4.3. Testing pRM45(Amp^R) for Contaminating λ DNA

In the transformation assay used to isolate the host Rpl8 strain (Maiti *et al*, 1991), a plasmid construct (pMR45) constitutively expressing *P* from the p_R promoter was used. This plasmid was reported as being lethal to wild type host cells upon transformation. The researchers propagated the construct by growing the plasmid on host cells lysogenic for λ. The CI repressor produced by the prophage inhibited expression of P protein from pMR45, allowing the lysogenic cells to be transformed with pMR45, whereas a non-lysogenic cell would not survive transformation.

When we received this plasmid construct from Dr. N.C. Mandal, it was successfully transformed into wild type 594 cells (data not shown). Personal communication with Dr. Mandal over this puzzling result led to the suggestion that their plasmid preparations of pMR45, made by growing on λ lysogens, were always contaminated with λ DNA. Dr. Mandal proposed that our survivor transformants contained not only the pMR45 plasmid, but that they had also obtained a contaminating λ prophage (*i.e.* are now lysogenic). I decided to test the 594[pMR45] cells for λ immunity.

3.1.6.4.3.1. Functional Immunity Assay

The Functional Immunity (FI) assay (Hayes and Hayes, 1986) was designed to test for λ immunity within a host cell. A 0.3 mL aliquot of W3350(λ*imm*434T) cells, 0.1 mL of λ*imm*434*cI*$^-$ free phage and 3 mL of molten top agar were mixed and poured onto a TB+Amp plate and allowed to solidify. Host cells to be tested for λ immunity (*i.e.* 594[pMR45] cells) were stabbed into the overlay, and the plates were incubated

overnight at 30°C. The next day, the plates were analyzed for areas of cell lawn lysis surrounding the stabbed cells. Cell lawn lysis is an indicator of marker rescue between the *imm*λ stabbed cell and the *imm*434 free phage, with the recombinant *imm*λ phage being hetero-immune to the W3350(λ*imm*434T) cells in the lawn. The heteroimmune phage would now be capable of forming a plaque on the cell lawn (*i.e.* area of lysis adjacent to stabbed cell).

When several single colonies of the two 594[pMR45] isolates were tested in the FI assay, all of the stabbed colonies were surrounded by areas of lysis (data not shown), indicating that the stabbed 594[pMR45] cells contained λ immunity; meaning that they contained a λ DNA fragment consisting of at least the *imm*λ region (*cI* gene, O_L and O_R operator sites).

3.1.6.4.3.2. Cross Streaking Assay for Immunity

The 594[pMR45] cells were then tested for their ability to grow past streaks of λ*cI*72 and λvir phages, as described in section 2.5.2. The two isolates of 594[pMR45] were able to grow through λ*cI*72 at 30°C, demonstrating resistance to an *imm*λ phage. The 594[pMR45] cells were not able to grow through the λvir phage (data not shown), demonstrating that they did not contain a mutation preventing λ attachment and infection. These results, taken together, suggest that the 594[pMR45] cells express λ immunity, *i.e.* contain a λ DNA fragment.

Both the functional immunity and cross streaking assays indicated that my 594[pMR45] cells contain λ immunity. The pMR45 plasmid (Maiti *et al*, 1991) does not contain DNA capable of producing λ immunity (*i.e.* no *cI* gene). These results

suggest that the cells contain a λ prophage in addition to the pMR45 plasmid. What effect this could have during the P-Interference assay remains to be determined.

3.1.6.4.3.3. Testing pMR45 Plasmid Preparation For Infectious λ Phage Particles

In an attempt to understand how my 594[pMR45] cells had acquired λ immunity, I wondered if the pMR45 plasmid preparation received from Dr. Mandal contained contaminating λ infectious phage particles. The 5 μl plasmid aliquot remaining from the preparation received from Dr. Mandal was plated on 594 host cells at 37°C, but no plaques were seen (data not shown). However, a very low titer of contaminating phage particles would not have been seen in this assay, since only 5 μl of the plasmid preparation was tested. It remains to be seen if a larger aliquot would have revealed the presence of phage particles.

3.2. INHIBITION PHENOTYPE SPECIFIC TO *rep*λ PHAGE DEVELOPMENT

3.2.1. Background Data / Rationale for Study

Previous studies revealed that *E. coli* cells transformed with plasmid pHB25, containing about 25% of the λ genome (including genes *sieB, N, rexB, rexA, cI*[ts]857*, cro, cII* and the N-terminal fragment of gene *O*, including *ori*λ), reduced the ability of infecting hetero-immune (*i.e.* phage carrying a different *imm* region, consisting of the *cI* repressor gene and the binding sites O_L and O_R) λ phage to form plaques (Bull, 1995). A similar observation had been made by Rao and Rogers (1978) with their plasmid pKC10, containing the identical λ sequence. Deletion derivatives of pHB25 were produced to determine which λ sequences on pHB25 were responsible for the observed inhibitory phenotype, which we abbreviate herein to "IP". The IP effect was seen with plasmids pHB26 and pHB27, (both $rop^+OOP^+ori^+$) and with pHB27R (OOP^+ori^+). All four plasmids share λ DNA sequences encoding part of gene *cII,* the proposed inceptor site *ice* (Hobom and Lusky, 1979a), and the adjacent t_O-*oop*-p_O through the N terminal end of gene *O*, including ITN4-AT (*ori*λ). They are deleted for *cro* and the p_R promoter, so that they do not express genes *cII* and *O*.

Harold Bull determined that plasmids containing DNA sequences from t_O-*oop*-p_O-*ori*λ retained the ability to inhibit homo- and hetero-immune phage development (IP) (Bull, 1995), however, these observations were never published and the problem was never fully documented. I undertook a re-examination of the plasmid elements and phage targets participating in the IP, with the goal of elucidating a mechanism.

3.2.2. Hypothesis for the Inhibition Phenotype

My first hypothesis is that the IP plasmids are acting as competitor *rep*λ origins, and are thus interfering with incoming *rep*λ phage DNA replication initiation. My second hypothesis is that OOP RNA, acting as an accessory element of the λ origin, increases the competitiveness of the IP plasmid.

3.2.3. IP Plaque Assay

Because several hetero-immune lambdoid phages presumably contain the same replication initiation proteins *O* and *P,* and *ori*λ, collectively termed "*rep*λ", I decided to determine if the IP was directed to *rep*λ DNA replication initiation. The efficiency of plating (EOP) was assayed for phages λ*cI*857 (*rep*λ) and λ*cI*857(*18,12*)P22 (*rep*P22) on *E. coli* cells containing derivatives of the IP plasmid pHB27 ($rop^+OOP^+ori^+$), Fig. 3.13 and Table 3.14. Efficiency of plating was calculated as the phage titer on cells containing IP plasmids divided by the phage titer on 594 cells. The *rep*P22 phage had an EOP near 1.00 on all host cells, and was thus defined as IP insensitive. The plating efficiency of the *rep*λ phage on hosts with plasmids carrying λ DNA segments is shown in Table 3.14. λ*cI*857 plated with an efficiency near 1.00 on 594[pBR322] cells, indicating that the presence of a ColE1 plasmid is not responsible for the IP. λ*cI*857 plated with an efficiency near 1.00 on 594[pHB29] (OOP^+) cells, indicating that the IP is not caused simply by OOP RNA over-expression. Cells containing pHB28 (ori^+) slightly inhibited the plating efficiency of λ*cI*857 (EOP = 0.30), indicating that *ori*λ alone has a minimal effect on the IP. The plasmid pHB27Rp_O^- contains the entire *oop* through *ori*λ DNA sequence, but the p_O promoter has been mutationally inactivated (the -10 region

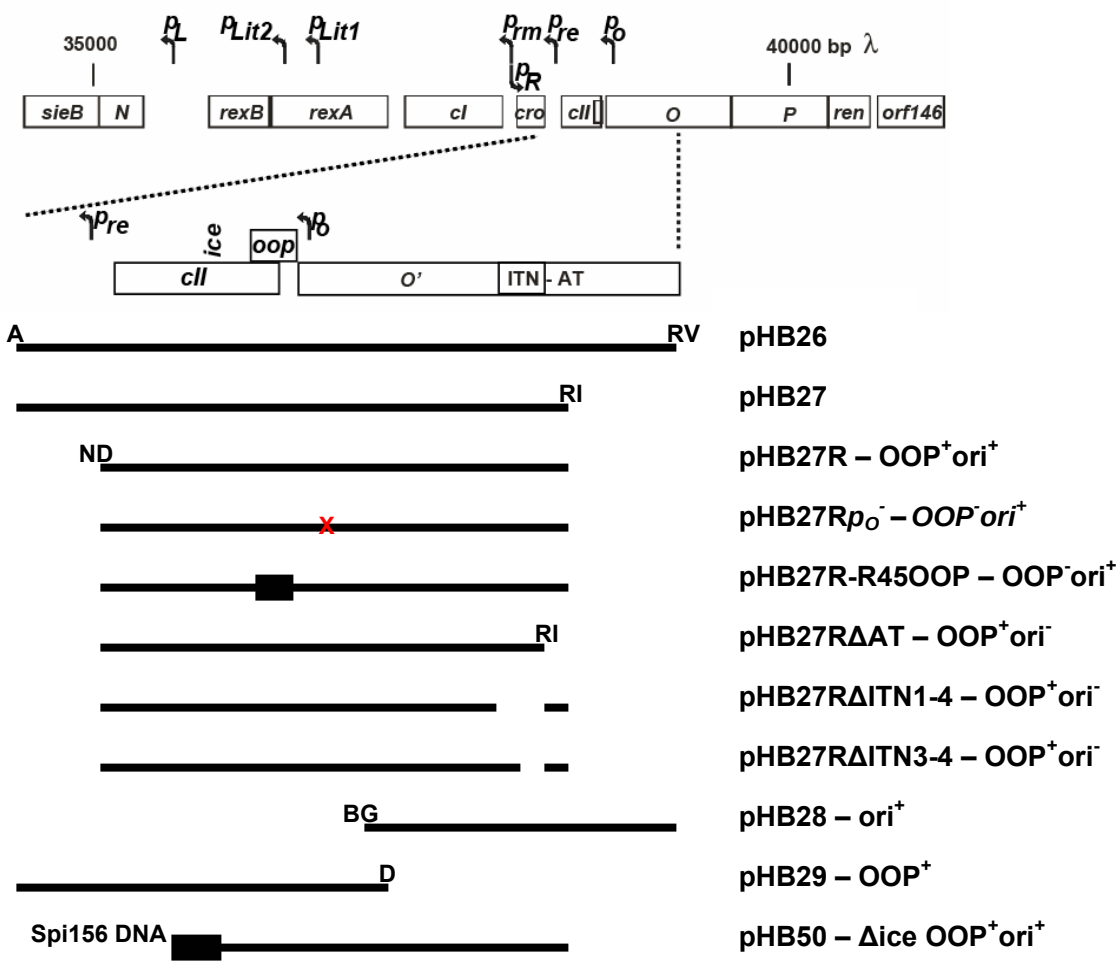

Figure 3.13. Inhibition Phenotype Plasmid Maps. All plasmids were constructed using a pBR322 backbone. Plasmid constructs containing the letter R in the name have been deleted for the plasmid copy control element *rop*. pHB26, pHB27, pHB28, pHB29 and pHB50 are all *rop*$^{+}$. A=*Aat*II; ND=*Nde*I; BG=*Bgl*II; D=*Dra*I; RI=*Eco*RI; RV=*Eco*RV.

Table 3.14. An Inhibition Phenotype Specific to the Plating of *rep*λ Phage Defined by Efficiency of Plating (EOP) at 30°C

λ*cI*857 infecting phages [b]	Efficiency of plating on 594 host cells with indicated plasmid +/- standard error [a]						
	None	pBR322	pHB29 OOP$^+$	pHB28 *ori*λ$^+$	pHB27Rp_O^- OOP$^+p_O^-$*ori*λ$^+$	pHB27R OOP$^+$*ori*λ$^+$	pHB50 *Δice*OOP$^+$*ori*λ$^+$
rep(*O-P*)λ	1.00 [c]	0.89 +/-0.13	0.99 +/-0.11	0.30 +/-0.03	0.34 +/-0.02	<0.00001	<0.00001
rep(*18,12*)P22	1.00	1.04 +/-0.07	0.70 +/-0.09	0.72 +/- 0.04	1.02 +/-0.08	0.93 +/-0.04	1.05 +/-0.12

[a] Efficiency of plating is defined by phage titer on strain 594[indicated plasmid] / titer on 594.
A 0.3 mL aliquot of fresh overnight stationary phase cells and 0.1 mL of infecting phage and 3 mL of molten TB top agar were poured onto a TB plate and incubated overnight at 30°C. Resulting plaques were counted and EOP was calculated.
[b] Infecting phages are λ*cI*857 and λ*cI*857(*18,12*)P22.
[c] Results are presented as the average of at least 2 individual trials. Standard errors for values < 0.00001 were < 1.0×10^{-6}.

sequence ATTAT has been replaced with GCGCG using Horton's SOEing PCR mutagenesis technique as described in Section 2.1.3.2), preventing transcription from p_O. This plasmid is phenotypically OOP$^-$ori$^+$. Cells containing pHB27Rp_O^- slightly inhibited plating by λ*cI*857 (EOP = 0.34), similar to effect caused by pHB28 (also OOP$^-$ori$^+$), suggesting again that the presence of *ori*λ alone (*i.e.* without simultaneous *oop* transcription from p_O) slightly inhibits plating of infecting λ*cI*857 phage. The two OOP$^+$ori$^+$ plasmid constructs (pHB27R and pHB50) drastically inhibited λ*cI*857 plating efficiency (EOP <0.00001). Permitting OOP RNA transcription from p_O increased inhibition towards a *rep*λ phage by 34000-fold; while permitting OOP transcription had no effect on the plating of a *rep*P22 phage. These results suggested that the IP requires the simultaneous presence of *ori*λ and the expression of OOP RNA (or at least transcription from p_O) and that the IP is targeted towards *rep*λ phage DNA replication initiation.

3.2.4. IP Prophage Induction Assay

Lysogens of λ*cI*[ts]857(*18,12*)P22 and λ*cI*[ts]857 were induced in the presence of plasmids pHB27R (OOP$^+$ori$^+$), pHB27Rp_O^- (*OOP$^+p_O^-$ori$^+$)*, pHB28 (ori$^+$) and pHB29 (OOP$^+$) to determine if the IP blocked phage morphogenesis and cell lysis resulting from de-repression of the prophage copy integrated within the host cell chromosome, Fig. 3.14. Lysogenic cells in the early log phase of growth were removed from 30°C, swirled in a 50°C water bath for 15 seconds and then placed in a 42°C water bath to induce the resident prophage. Absorbance readings (A_{575nm}) were taken at 30 minute intervals to monitor cell lysis as an indirect measure of prophage induction.

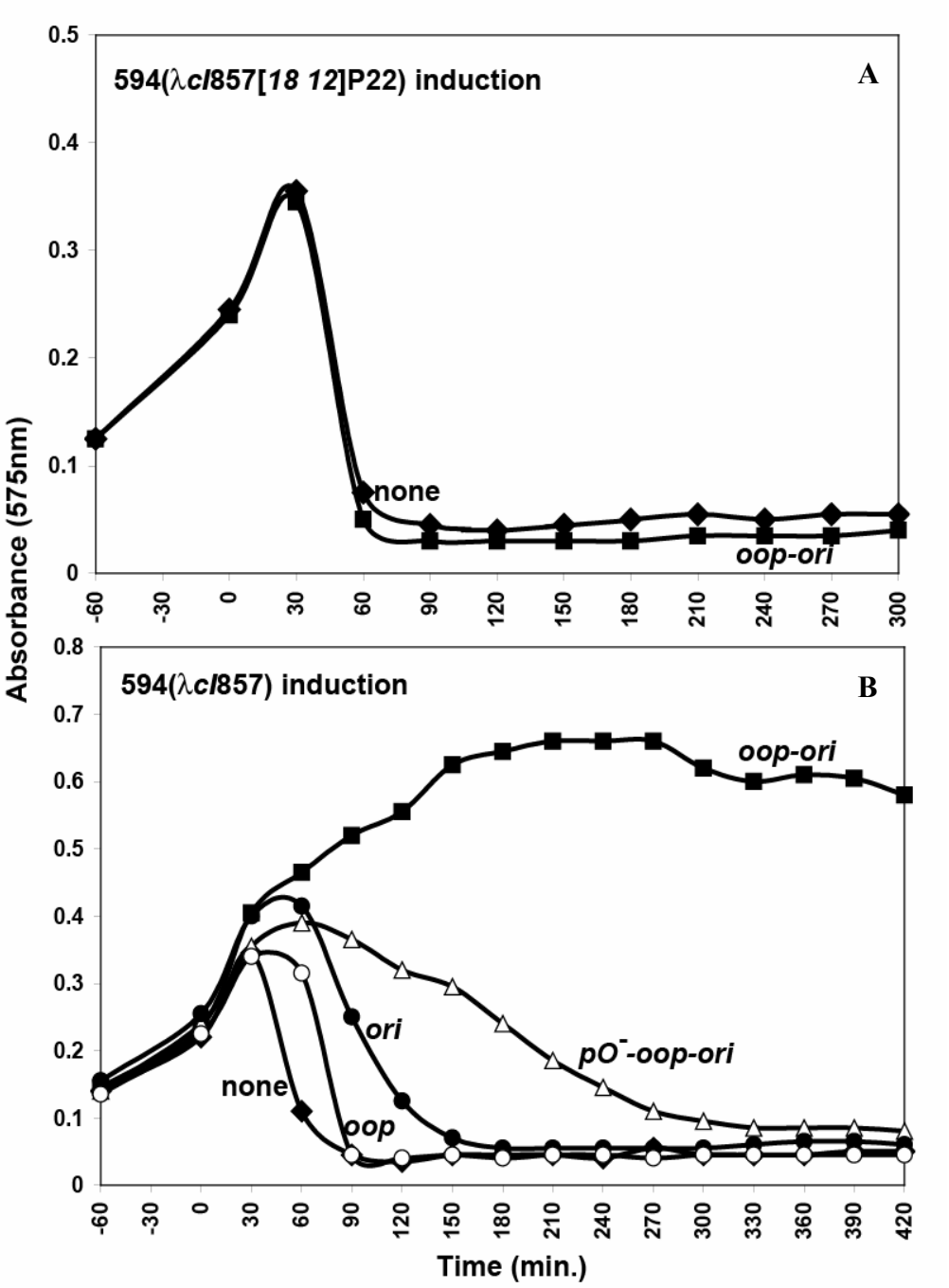

Figure 3.14. Thermal Induction of *rep*λ and *rep*P22 λ*cI*857 Prophages. Lysogenic cultures were grown at 30°C and prophages were thermally induced by shifting culture flasks from 30°C to 42°C at time 0. The results represent the averages for 2 independent assays. The standard error values ranged from 0.005-0.145. None indicates culture 594; oop-ori indicates 594[pHB27R] (OOP^+ori^+); pO^--oop-ori indicates 594[pHB27Rp_O^-] ($OOP^+p_O^-ori^+$); ori indicates 594[pHB28] (ori^+); and oop indicates 594[pHB29] (OOP^+). Curves represent the averages of at least two independent trials. Standard error values, not presented, were ≤ 0.15.

None of the four plasmids prevented the induction, morphogenesis, or the timing of phage-encoded lysis of cells with the λ*cI*[ts]857(*18,12*)P22 prophage, Fig. 3.14A. The results are only shown for plasmid pHB27R (OOP^+ori^+), but the curves are essentially super-imposable. Prophage induction occurred at time 0. Cell growth continued for 30-60 minutes, at which point fully developed, mature phage particles began to lyse the cells. Within 30 minutes, absorbance values plummeted, with the majority of cells having been lysed by the escaping phage particles. Fig. 3.14B shows that vegetative phage development and cell lysis resulting from the induction of the *rep*λ prophage λ*cI*857 was markedly inhibited by the plasmid pHB27R (OOP^+ori^+), and while cell lysis did eventually occur in host cells containing the other plasmid constructs, it was increasingly delayed by plasmids pHB29 (OOP^+), pHB28 (ori^+), and pHB27Rp_O^- ($OOP^+p_O^-ori^+$), respectively.

These induction results support the conclusions made from the plaque assays, *i.e.* that temporal phage development is arrested for a single *rep*λ phage copy present in cells with plasmids containing the t_O-*oop*-p_O through *ori*λ sequence, and that both OOP transcription and the close proximity of *ori*λ on the fragment are required for the IP. The OOP-ori components of the plasmid are interfering with *rep*λ phage development, and are likely inhibiting *ori*λ-dependent DNA replication initiation.

3.2.5. Variation in Susceptibility of *rep*λ Phages to the IP

It has been demonstrated that the *rep*λ phage, λ*cI*857, was susceptible to the IP, while the otherwise isogenic *rep*P22 phage, λ*cI*857(*18,12*)P22 was insensitive, Table 3.14 and Fig. 3.14. It remained to be seen if all *rep*λ phages were equally sensitive to

the plasmid-dependent inhibition of phage development.

3.2.5.1. IP Plaque Assay For *rep*λ Phages

The two plasmids previously shown to inhibit λ*cI*857 plating (Fig. 3.13 and Table 3.14), pHB27R (OOP$^+$ori$^+$) and pHB50 (Δ*ice*OOP$^+$ori$^+$), were tested for their ability to inhibit other *rep*λ phages in the plaque assay.

594 cells with either pHB27R (OOP$^+$ori$^+$) or pHB50 (Δ*ice*OOP$^+$ori$^+$) were infected with homo- or hetero-immune *rep*λ phages, Fig. 3.15. Phages λ WT (*cI*$^+$), λ*cI*72, λ*cI*90*c*17 and various λo_R mutants (λse100a, λse101b and λse109b; isolated by Hayes and Hayes, 1986; produce a CI-defective phenotype and likely inhibit p_{RM} transcription, while possibly enhancing p_R transcription) were as sensitive to the IP at 30^{o}C as was λ*cI*857 (Table 3.14), suggesting that the inhibition of *rep*λ DNA replication was not modulated by the λ CI repressor, or by presumed variations in transcription arising from p_R or by p_R-independent transcription arising from the *c*17 mutation (Rosenberg *et al*, 1978). The *rep*λ phages λ*imm*434*cI*, λ*imm*21*cI* and λvir were only slightly sensitive to the IP, as indicated by the formation of pfu at relatively high efficiency on 594[pHB27R] (OOP$^+$ori$^+$) and 594[pHB50] (Δ*ice*OOP$^+$ori$^+$) cells, Fig. 3.15. Another *rep*λ phage, λΔ*cII* (Oppenheim *et al*, 2004), deleted for *oop*, also plated at relatively high efficiency on 594[pHB27R] (OOP$^+$ori$^+$) cells (data not shown). The sequence for *oop* in phage 434 was reported to have three base alterations with respect to the *oop* sequence in λ (Grosschedl and Schwarz, 1979) and phage λ*imm*21 has a hybrid 21/λ *oop* sequence (Daniels *et al*, 1983). It would appear that alteration of DNA sequences adjacent to *ori*λ can profoundly influence the sensitivity of an infecting phage

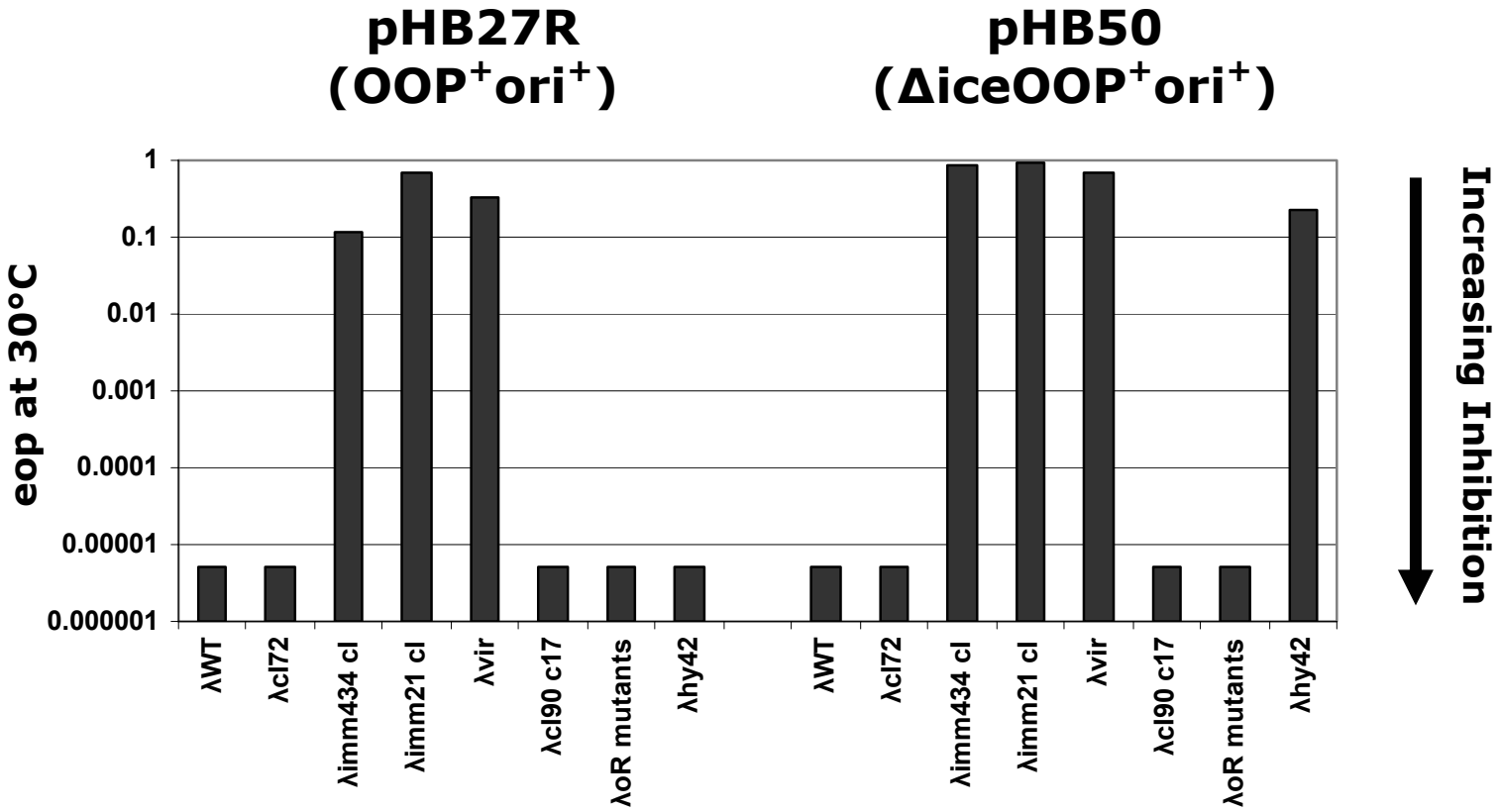

Figure 3.15. Variation in Susceptibility of *repλ* Phages to the IP. A 0.3 mL aliquot of fresh overnight stationary phase cells (grown in TB+Amp) were mixed with 0.1 mL of test phage and 3.0 mL of molten top agar and poured onto a TB plate. Plates were incubated overnight at 30°C and resulting pfu were counted. EOP was calculated as the titer on strain 594[indicated plasmid]/titer on 594. The results represent the average of at least two independent assays. Standard errors values for all phage showing inhibition were $< 1.0 \times 10^{-6}$ and the values for phage escaping IP ranged from 0.04-0.26.

to the IP.

A *rep*Φ80:*rep*λ phage, λhy42, in which the N-terminal end of *O* is reported to be replaced by Φ80 DNA (Moore *et al,* 1979; Moore *et al*, 1981) showed an interesting result in that 594[pHB27R] (OOP^+ori^+) and 594[pHB50] (Δ*ice*OOP^+ori^+) cells inhibited phage plating differently, Fig. 3.15. λhy42 was completely inhibited on 594[pHB27R] (OOP^+ori^+) cells, but was able to plate on 594[pHB50] (Δ*ice*OOP^+ori^+) cells at relatively high efficiency. This led me to examine any differences between these two plasmids, which might explain the plating discrepancies.

Both constructs are OOP^+ and ori^+; however, pHB27R (OOP^+ori^+) is rop^- and ice^+, while pHB50 (Δ*ice*OOP^+ori^+) is rop^+ and ice^-. Since the Rop protein is important in controlling ColE1 plasmid copy number, pHB27R (OOP^+ori^+) should have a higher copy number than pHB50 (Δ*ice*OOP^+ori^+). Phage EOP and plaque size on 594[pHB27R] (OOP^+ori^+), 594[pHB50] (Δ*ice*OOP^+ori^+) and 594[pHB27] ($rop^+OOP^+ori^+$) cells were measured to determine if the difference in λhy42 inhibition could simply be explained by plasmid copy number, or if the proposed inceptor site, *ice*, was important for the IP, Table 3.15. It was shown that pHB27 ($rop^+OOP^+ori^+$) usually inhibited phage plating in a similar manner to pHB27R (OOP^+ori^+). It was also determined that phage growing on 594[pHB27] ($rop^+OOP^+ori^+$) cells produced plaques intermediate in size between pHB27R (OOP^+ori^+) and pHB50 (Δ*ice*OOP^+ori^+) cells, with pHB50 (Δ*ice*OOP^+ori^+) allowing the largest plaques to develop (data not shown). These results suggest that although plasmid copy number may play some role in the IP, there remains something specific to pHB50 (Δ*ice*OOP^+ori^+) which allows λhy42 to be less inhibited than on pHB27R (OOP^+ori^+); likely the absence of *ice*, the inceptor site, proposed as being an

Table 3.15. Effect of Plasmid Copy Number Regulation by Rop and the Proposed Inceptor Site on the IP

Infecting Phage	Phage EOP at 30°C on indicated host strain [a]			
	594	**594[pHB27R]** **(OOP^+ori^+)**	**594[pHB50]** **($\Delta ice OOP^+ori^+$)**	**594[pHB27]** **($rop^+OOP^+ori^+$)**
λwt	1.00	$<1.0 \times 10^{-5}$	5.0×10^{-5}	$<1.0 \times 10^{-5}$
λ*cI*72	1.00	$<1.0 \times 10^{-5}$	$<1.0 \times 10^{-5}$	$<1.0 \times 10^{-5}$
λ*cI*857(*18,12*)P22	1.00	0.95	0.88	0.79
λhy42	1.00	$<1.0 \times 10^{-5}$	0.20	$<1.0 \times 10^{-5}$
λ*imm*434*cI*	1.00	0.03	0.49	0.18
λ*imm*21*cI*	1.00	0.75	0.80	0.77
λvir	1.00	0.34	0.77	0.66

[a] Efficiency of plating is defined by phage titer on strain 594[indicated plasmid] / phage titer on 594.
A 0.3 mL aliquot of fresh overnight stationary phase cells and 0.1 mL of infecting phage and 3 mL of molten TB top agar were poured onto a TB plate and incubated overnight at 30°C. Resulting plaques were counted and the EOP was calculated.

alternate start site for DNA synthesis when *ori*λ-dependent replication is blocked (Lusky and Hobom, 1979a).

3.2.5.2. IP Plaque Size Variation of *rep*λ Phages

The plaque assay revealed that infecting phage plaque size was significantly influenced by the presence of the plasmids used in Table 3.14. Plaque size is a qualitative measure of phage development or burst; phages impaired for development, particularly for replication, show a corresponding reduction in plaque size (Hayes *et al*, 2005). Therefore, I determined the ability of the various plasmids used in Table 3.14 to influence infecting phage plaque size. Phage plaque sizes were determined using a tissue culture (inverted) microscope at 4X magnification with an eyepiece grid. Each grid interval was 0.45 mm at the 4 X magnification. Plaque diameters were measured as grid units, *i.e.*, grids/plaque. Approximately 30 plaques were measured per assay phage (for phages λ, λ*cI*72, λ*cI*857, λvir, λ*imm*434*cI*$^{-}$, λ*imm*21cI^{-}, λ*cI*857(*18,12*)P22, λ*cI*90c17, λse100a, λse101b and λse109b) on each of the host strains, and the average plaque diameter and standard error were determined. All assays for a given phage were performed in parallel on each of the host strains and representative results for five of these phages are shown in Fig. 3.16.

Directly quantitating plaque size variation yielded further information regarding *rep*λ phage susceptibility to the IP. While λvir, λ*imm*434*cI*$^{-}$ and λ*imm*21*cI*$^{-}$ all formed plaques on cells with the inhibitory plasmids, they are, nevertheless, subject to replicative inhibition, as their plaque sizes are significantly reduced by the *rep*λ-inhibitory plasmids, Fig. 3.16.

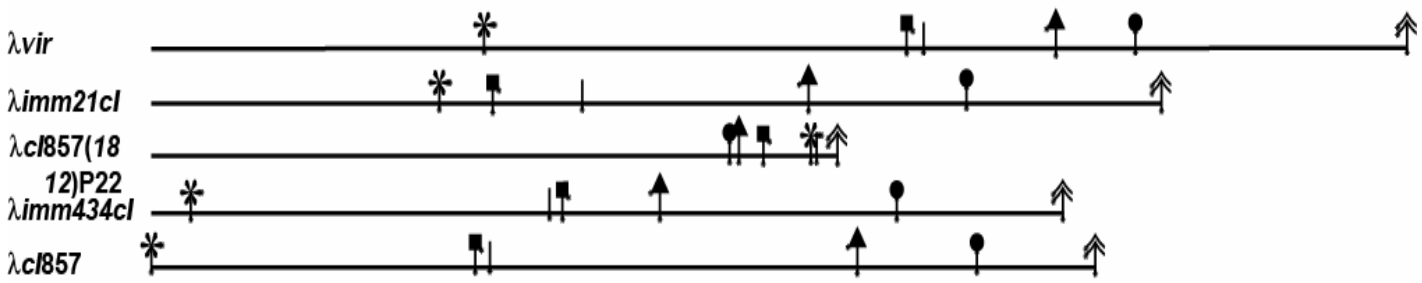

Figure 3.16. Effect of λ Plasmids on Infecting Phage Plaque Size. Plaque sizes were measured using a tissue culture microscope at 4 X magnification. Relative plaque diameter is indicated by the length of the line, from left to right. Notations above the line indicate the stop point for each plasmid variant, *i.e.* the further the symbol is to the right, the larger the plaque. Square shape indicates no plasmid; triangle indicates pBR322; asterix indicates pHB27R (OOP^+ori^+); square indicates pHB27Rp_O^- ($OOP^+p_O^-ori^+$); vertical line indicates pHB28 (ori^+); double ampersand indicates pHB29 (OOP^+).

In every phage assayed for plaque size (plaque size was not determined for λhy42 or λΔ*cII*), each phage formed the largest plaques on host cells 594[pHB29] (OOP$^+$). The *rep*P22 phage λ*cI*857(*18,12*)P22 formed plaques of about equal size on all tested host strains. The *rep*λ phages λvir, λ*imm*21*cI*$^-$ and λ*imm*434*cI*$^-$, which appeared to escape the IP in the plaque assay (Fig. 3.15), were nevertheless found to form smaller plaques on 594[pHB27R] (OOP$^+$ori$^+$) cells, with λ*imm*434*cI*$^-$ giving barely visible (pin-prick) plaques, further supporting the observation that *rep*λ phages are susceptible to the IP. Phages that were fully sensitive to the IP in 594[pHB27R] (OOP$^+$ori$^+$) cells, *e.g.* λ*cI*857 (see Fig. 3.12, 3.13 and Table 3.6), were also reduced in plaque size on host cells containing *ori*λ, *i.e.* 594[pHB28] (ori$^+$) and 594[pHB27Rp_O^-] (*OOP$^+p_O^-$ori$^+$)*. The results for phages λwt, λ*cI*72, λse101b and λse109b were nearly identical in size and pattern of inhibition to the results presented for λ*cI*857, while phages λ*cI*90*c*17 and λse100a were about 20% larger (data not shown).

3.2.5.3. Phage Sequencing Data From *ice* Through *ori*λ

The IP plaque assay indicated that various *rep*λ phages have variable susceptibilities to the IP. Since the plasmids inhibiting phage development required the presence of *ori*λ and active transcription from p_O, it was hypothesized that the t_O-*oop*-p_O through *ori*λ region on the infecting phage genomes might be the target for the IP. It was hypothesized that the phage variability to the IP might be due to sequence variation in this region of the genome. It was decided to sequence the various phages utilized in the IP assay for any sequence changes or mutations which might account for variation in susceptibility to the IP.

The phages λpapa (wt), λ*cI*857, λ*cI*72, λvir, λ*imm*434*cI*⁻, λ*cI*90c17, λse100a, λse101b and λse109b were all amplified and sequenced with primers L22 (λ38517-38534) and R9+1 (λ39191-39175). DNA sequence data was obtained from the C-terminal portion of the *cII* gene (including *oop* and *ice*) through the AT rich region of *ori*λ in gene *O*. All of the above phages contained wt λ DNA sequence in this region (λ DNA sequence was obtained from the NCBI nucleotide database, Genbank accession number NC001416). Phage 434's *oop* gene (the above λ*imm*434 phage is a hybrid containing the immunity region of phage 434) contains 3 point mutations compared to λ. The sequencing data shows that the λ*imm*434*cI*⁻ hybrid contains the λ *oop* gene, *i.e.* the gene showed 100% sequence homology to λ.

λ*imm*21*cI*⁻, a hybrid between phages 21 and λ, was amplified and sequenced with primers LΦ21cII (Φ21 38513-38530) and R9+1 (λ39191-39175). λ*imm*21*cI*⁻ contains a hybrid *oop* gene, with the C-terminal portion being phage 21 sequence, and the N-terminal portion being λ sequence. Sequence analysis indicated that the hybrid *oop* gene was three nt longer than the λ *oop* gene and contained five nt changes at the C-terminal end of the gene. The 21 and λ *oop* sequences are remarkably similar, only differing in five positions. Nucleotide changes were seen at positions 38599 and 38613; and single nt insertions at 38615, 38620 and 38621, Fig. 3.17. The results indicate that the sequence divergence point occurred at λ38619. λ*imm*21*cI*⁻ also contained an C to T base change in the N-terminal region of gene *O*, at 39033, immediately before ITN-1, with no subsequent aa alteration.

λ*cI*857(*18,12*)P22, a hybrid between phages P22 and λ, was sequenced with primers L22 (λ38517-38534) and RP22-18 (P22 sequence 33000-32983).

Figure 3.17. Sequence Alignment of the *oop* Genes of Phages Φ21, λ*imm*21*cI⁻* and λ. The Φ21 and λ sequences were obtained from the NCBI genome database; Genbank accession numbers AJ237660 and NC001416, respectively. The λ*imm*21*cI⁻* phage was sequenced at NRC-PBI; both the *l*- and *r*-strands were sequenced. The dotted vertical line indicates the hybridization point in the hybrid phage λ*imm*21*cI⁻*, with the phage containing Φ21sequence on the left side, and λ sequence on the right. The sequence divergence point occurs within the coding sequence for the *oop* gene, which begins at λ38675 and ends at λ38599. Sequences identical in all three phages are indicated in black. Red sequences indicate the sites where Φ21 and λ*imm*21 phages show sequence differences from phage λ (shown in blue). The hybrid *oop* gene consists of λ sequence from 38675-38619, followed by Φ21 sequence from 38620-38596. The λ*imm*21*cI⁻* gene contains 2 base pair changes compared to the λ *oop* gene (at 38599 and 38613). The hybrid gene is also 3 nt longer than the λ *oop* gene, due to the insertion of 3 nt.

λ*cI*857(*18,12*)P22, obtained from S. Hilliker as a λ*cI*857 phage containing the phage P22 replication genes *18* and *12* in place of the λ replication genes *O* and *P* (phage hy106), has never been sequenced in order to specifically identify where the hybrid break occurred and whether the phage contained *orf48* from phage P22. Sequence analysis, Fig. 3.18, indicated that this phage contains λ DNA from *ice* through to 38667, including the entire λ *oop* gene, except for the first eight nt. The first eight nt of *oop* are phage P22 encoded. The third and seventh *oop* bases contained nt changes as compared to the λ *oop* sequence. Phage P22 sequence begins at P22 base 32331, meaning that the hybrid phage encodes the *orf48* sequence, followed by gene *18*.

λhy42 was proposed to be a hybrid between phages Φ80 and λ, reported to contain immΦ80 and a hybrid Φ80:λ *O* gene (Furth, 1978). The primer pair LΦ80cII (NCBI # AJ237660 Φ80 sequence 4139-4155) and R9+1 (λ39191-39175) were used in an attempt to amplify this phage from Φ80*cII* through *ori*λ. Several attempts failed to produce a PCR product. However, the λ primers L22 and R9+1 were able to produce a PCR product, which when sequenced, indicated wt λ sequence from *ice* through *ori*λ. These results demonstrated that the λhy42 lysate utilized in the IP plaque assay was not actually a Φ80/λ hybrid, as formerly believed. I had a very hard time finding a viable lysate of this phage, as most of our preparations had died. So it seems that we no longer have any λhy42 preps left for further study. This *rep*λ phage, containing wt λ sequence from *ice* through *ori*λ, is intriguing as it was the only tested phage that showed plating differences on 594[pHB27R] and 594[pHB50] host cells.

In summary, the only two phages with any sequence variation between *ice* and *ori*λ were λ*imm*21*cI*⁻ and λ*cI*857(*18,12*)P22. The *rep*P22 phage λ*cI*857(*18,12*)P22 has

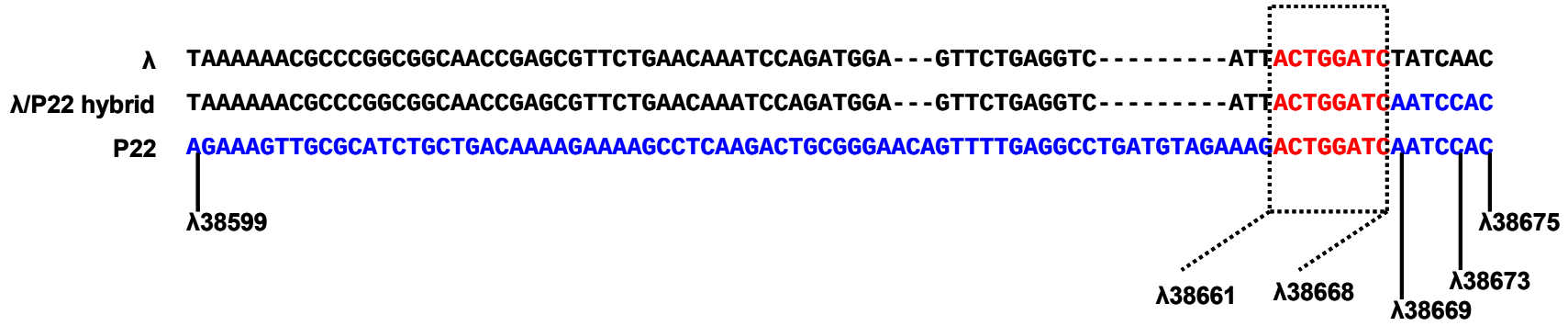

Figure 3.18. Sequence Alignment of the *oop* Genes of Phages λ, λ*cI*857(*18,12*)P22 (called λ/P22 hybrid in figure) and P22. The P22 and λ sequences were obtained from the NCBI genome database; Genbank accession numbers NC002371 and NC001416, respectively. The λ*cI*857(*18,12*)P22 phage was sequenced at NRC-PBI; while both the *l*- and *r*-strands were sequenced, only the *l*-strand data is presented. The dotted vertical box indicates the sequence divergence point in the hybrid phage λ*cI*857(*18,12*)P22, with the phage containing λ sequence on the left side, and P22 sequence on the right. Both phages contain identical sequence in the boxed region, so it is impossible to determine which parental strain the hybrid received this segment from. The sequenced divergence point occurs within the coding sequence for the *oop* gene, which begins at λ38675 and ends at λ38599. The hybrid *oop* gene consists of P22 sequence from 38675-38669, followed by λ or P22 sequence from 38668-38661 and λ sequence from 38660-38599. The λ*cI*857(*18,12*)P22 *oop* gene contains 2 base changes compared to the λ *oop* gene (at 38669 and 38673).

already been shown to be insensitive to the plasmid-mediated replicative inhibition. Interestingly, λ*imm*21*cI⁻* showed the least sensitivity to plasmid-mediated replicative inhibition of any *rep*λ phage tested, suggesting that the actual OOP RNA sequence may be important in the IP. Why the λ*imm*434*cI⁻* phage, containing a wt λ *oop* gene, was able to partially escape inhibition is not yet understood.

3.2.6. The IP Can be Bypassed in Multiply Infected Cells

Because inhibition in phage development was directed to *rep*λ phages, but not to the *rep*P22 phage for both the plaque assay and the prophage induction assay, I postulated that the inhibition was directed to phages that expressed λ genes *O* and *P,* which act at *ori*λ within *O*. This suggested that the inhibition is directed to λ DNA replication initiation from *ori*λ, *i.e.* the theta mode of λ replication. Other studies undertaken within this laboratory (C. Hayes and S. Hayes, unpublished data with host strains containing the *dnaB*grpD55 mutation) suggested that *ori*λ-specific replication initiation, dependent upon P-DnaB interaction, could be bypassed in multiply infected cells where phage replication could be driven by intermediates derived from homologous recombination between multiple infecting phage genomes. Therefore, I asked if the IP was suppressed in multiply infected cells. I compared these phage burst results to those for *E. coli* cells with a *dnaB* mutation which prevents its ability to participate in λ replication initiation, *i.e.* the *dnaB*grpD55 allele (Hayes *et al,* 2005).

The IP plaque assay in essence represented a situation where each aliquot of host cells (about $1x10^8$) was mixed with 10^3 or fewer phage, so that the possibility of a cell being infected with two or more phage particles was very low, *i.e.* at an moi of

less than or equal to 1.0. I hypothesized that increasing the moi could influence the ability of an infecting phage to replicate and grow vegetatively on cells exhibiting the IP. Phage bursts were measured for phage infections at an moi of approximately 5 and compared to infections at an moi of 0.01, where it is assumed that the majority of cells will not be multiply infected. Bursts were determined for the infecting phages λ*cI*857, previously found to be sensitive to the IP, and λ*cI*857(*18,12*)P22, previously found to be insensitive to the IP, Table 3.16.

3.2.6.1. W3350 *dnaB*grpD55 Phage Burst Assay

λ*cI*857(*18,12*)P22 produced a phage burst of 11.3 at an moi of 0.01 on W3350 cells, very similar to the burst seen at an moi of 5, demonstrating that this *rep*P22 phage was able to undergo a productive infection in W3350, regardless of the moi, Table 3.16. Phages with the rep region (genes *18* and *12*) of phage P22 encode their own DnaB helicase, *i.e.* the product of gene *12*, and so do not require the *E. coli* host *dnaB* gene product for replication (Taylor and Shizuya, 1981). The results in Table 3.16 show that phage λ*cI*857(*18,12*)P22 replication was indeed independent of the *E. coli* DnaB helicase, producing a phage burst of 17.1 at an moi of 0.01 on W3350 *dnaB*grpD55 cells and a very similar burst at an moi of 5. The *dnaB*grpD55 allele is not capable of supporting *ori*λ-dependent replication initiation (Hayes *et al,* 2005). I speculate that the two missense mutations arising within the *grpD*55 allele of *dnaB* (Table 3.12) influence the P-DnaB interaction required for phage λ replication initiation. The results in Table 3.16 indicate that λ*cI*857(*18,12*)P22 is able to replicate independently of the host replicative helicase, DnaB. The λ*cI*857 infection of W3350

Table 3.16. *Oriλ*-dependent DNA Replication Initiation can be Bypassed in Multiply Infected Cells

Host Strain	Infecting Phage [a]			
	λ*cI*857 [*rep*λ]		**λ*cI*857(*18,12*)P22 [*rep*P22]**	
	moi 5	**moi 0.01**	**moi 5**	**moi 0.01**
W3350	35.5 +/- 5.3 [b]	31.0 +/- 6.2	13.8 +/- 1.4	11.3 +/- 3.4
W3350*dnaB*grpD55	31.6 +/- 16.3	1.34 +/- 0.7	19.2 +/- 7.3	17.1 +/- 3.2
594	25.8 +/- 4.2	25.3 +/- 8.5	14.6 +/- 3.8	9.4 +/- 0.8
594[pBR322]	22.4 +/- 4.8	26.0 +/- 12.3	9.0 +/- 1.0	5.7 +/- 0.1
594[pHB27R] (OOP^+ori^+)	21.1 +/- 8.2	1.1 +/- 0.7	6.8 +/- 0.6	6.1 +/- 0.7
594[pHB27Rp_O^-] ($OOP^+p_O^-ori^+$)	27.3 +/- 7.2	19.8 +/- 6.6	10.9 +/- 0.4	6.9 +/- 2.2

[a] A fresh overnight culture of stationary phase cells (at approximately 2.0 x 10^9 cfu/mL) was washed and resuspended in Φ80 buffer. 0.2 mL of 0.01M $MgCl_2/CaCl_2$ and 0.1 mL of washed host cells were mixed (on ice) with an appropriate volume of phage in order to obtain an moi of either 5 or 0.01. The assay mix was held on ice for 15 min to allow for phage attachment, then transferred to 42°C and incubated for 10 minutes to allow infection to proceed. The mixture was spun in an Eppendorf tabletop centrifuge at 13000 RPM for 1.5 minutes and the pellet was resupended in Φ80 buffer. The wash step was repeated twice. The last pellet resuspension was done using prewarmed 42°C TB to a final volume of 0.4 mL. Half (0.2 mL) of the assay mix was used to inoculate 20 mL of TB (+ Amp where required) which was then incubated with shaking at 42°C. At 65 min and 110 minute intervals, aliquots were removed, diluted and plated to measure the number of phage released following cell lysis. The number of phage particles released per 0.2 mL original aliquot was calculated. The remaining 0.2 mL aliquot from the reaction mixture was spun once more and the supernatant was removed to a fresh tube. The number of free phage per 0.2 mL aliquot was calculated from this supernatant. The pellet was resuspended in 0.2 mL of prewarmed TB and the number of infective centers per 0.2 mL aliquot was calculated as the number of plaques per 0.2 mL.

[b] The results are expressed as phage burst (# phage particles released per infective center) +/- standard error. Each value represents the averages of at least two separate trials. Sample calculations are presented in Section 2.5.5.

at an moi of 0.01 was able to produce a phage burst of 31.0, Table 3.16, essentially identical to the burst seen on W3350 cells at an moi of 5. These results show the ability of λ*cI*857 to undergo a productive infection on W3350 cells, regardless of moi. λ*cI*857 was unable to produce a productive phage burst on the W3350 *dnaB*grpD55 cells at an moi of 0.01, burst of 1.34; whereas identical cells infected in parallel at an moi of 5 yielded a phage burst of 31.6, Table 3.16. The λ requirement of active DnaB helicase for *ori*λ-dependent replication initiation from a single phage chromosome was seen to be bypassed if the cells were multiply infected with the same phage in recombination proficient host cells. These results essentially duplicate and support the previous unpublished observations as reported above. Multiply infected cells have bypassed the requirement of a functional DnaB protein for *ori*λ-dependent replication initiation.

3.2.6.2. IP Phage Burst Assay

I have utilized the assay system illustrated in Table 3.16 to ascertain if the plasmid-dependent inhibition phenotype that was found to be directed towards *rep*λ phages, acts at the stage of *rep*λ DNA replication initiation. My hypothesis is that the IP plasmid pHB27R (OOP^+ori^+) will inhibit *rep*λ phage development at the stage of *ori*λ-dependent replication initiation and that plasmid-dependent *rep*λ phage inhibition will be suppressed (bypassed) when the inhibitory host cells are multiply infected. The hypothesis would be validated by an observation that the burst for singly infected cells is inhibited, but that multiply infected cells yield a burst equivalent to host cells without the *rep*λ inhibitory plasmids.

Results were compared for singly and multiply infected plasmid-containing cells with the *rep*λ phage λ*cI*857 and the *rep*P22 phage λ*cI*857(*18,12*)P22, Table 3.16. Phage λ*cI*857(*18,12*)P22 produced similar bursts at moi's of 5 and 0.01, within a factor of 1.6 on each of the host cell infections, Table 3.16, and was generally unaffected by the presence of the host mutation in *dnaB* or by the presence of any of the plasmids. The *rep*λ phage λ*cI*857 produced productive bursts upon infecting 594 and 594[pBR322] hosts at moi's of 5 and 0.01. Thus λ*cI*857 was able to undergo efficient vegetative growth when singly or multiply infecting 594 cells, and the plasmid pBR322 (the ColE1 plasmid originally used to construct the other plasmids) does not interfere with vegetative phage development, Table 3.16. λ*cI*857 was unable to produce a productive phage burst on singly infected 594[pHB27R] (OOP^+ori^+) cells (moi of 0.01 burst = 1.1); but when the same cells were multiply infected in parallel at an moi of 5, the λcI857 infection yielded a burst of 21.1. This result indicated that a *rep*λ phage was capable of suppressing plasmid-dependent *rep*λ phage inhibition by multiple phage infection, much as they overcame the *ori*λ-dependent replication initiation block in host cells containing the conditional *dnaB*grpD55 mutation. In contrast, λ*cI*857 was able to produce productive phage bursts in singly or multiply infected host cells containing a plasmid that was defective in plasmid-dependent *rep*λ phage inhibition, *i.e.* on 594[pHB27Rp_O^-] host cells, Table 3.16. Collectively, these results suggest that the IP is a form of plasmid-dependent *rep*λ replication initiation inhibition, which requires both the *ori*λ DNA sequence and RNA transcription from the promoter p_O.

3.2.7. Refining IP Plasmid Requirements

I have shown that cells with a small plasmid incorporating a short λ sequence fragment containing both t_O-*oop*-p_O (which must be transcribed from the promoter p_O) and *ori*λ DNA collectively confer vegetative developmental inhibition to single, but not multiply infecting *rep*λ phages, and also inhibit the development of an induced *rep*λ prophage, present as a single copy in the cell. This observation was qualitatively referred to as the Inhibition Phenotype (IP). It remains to be determined whether OOP RNA, the actual 77 base RNA product of t_O-*oop*-p_O transcription from p_O, or if all the parts of the *ori*λ sequence, *i.e.* both the four iteron sequences and the adjacent high AT rich sequence, participate in the IP. New plasmids were made to investigate the requirements of the t_O-*oop*-p_O and *ori*λ DNA sequences for the IP, illustrated in Fig. 3.13. All of the new plasmid derivatives were evaluated in the IP plaque assay, using the infecting phages λ*cI*857 and λ*cI*857(*18,12*)P22 and the results are described in Table 3.17.

3.2.7.1. pHB27R-R45OOP

In order to determine if transcription from p_O is essential for the IP, or if OOP RNA plays a specific role, it was decided to replace the nucleotide sequence of the *oop* gene with non-oop DNA. The *oop* gene is 77 nt long, with the C-terminal 31 nt being involved the formation of a self-terminating stem-loop structure. It was decided to retain the stem-loop structure in order to ensure that the new RNA would also be self-terminating. The first nt of the *oop* gene sequence (a rare G) was also retained. The *oop* bases 2-46 were replaced with a randomly selected DNA sequence, using the

Table 3.17. IP Plaque Assay for pHB27R t_O-*oop*-p_O and *ori*λ Derivatives

Host Strain	λ*cI*857 [a] [*rep*λ]	λ*cI*857(*18,12*)P22 [*rep*P22]	Inhibition Phenotype
594	1.00 [b]	1.00	No
594[pHB27R] (OOP+ori+)	<0.00001	1.26 +/- 0.01	Yes
594[pHB27R-R45OOP]	0.59 +/- 0.02	0.94 +/- 0.08	No
594[pHB27RΔAT]	<0.00001	1.01 +/- 0.05	Yes
594[pHB27RΔITN1-4]	1.14 +/- 0.08	1.16 +/- 0.13	No
594[pHB27RΔITN3-4]-2 wt *oop* gene	0.57 +/- 0.16	1.09 +/- 0.09	No
594[pHB27RΔITN3-4]-5 spontaneous *oop* gene mutation [c]	0.87 +/- 0.02	1.11 +/- 0.10	No

[a] Infecting phages are λ*cI*857 and λ*cI*857(*18,12*)P22.
A 0.3 mL of fresh overnight stationary phase cells and 0.1 mL of infecting phage and 3 mL of molten TB top agar were poured onto a TB plate and incubated overnight at 30°C. The resulting plaques were counted and EOP was calculated.
[b] Values are presented as efficiency of plating (defined by phage titer on strain 594[indicated plasmid] / titer on 594). Values represent the average of at least two individual trials. Standard error values are also presented. Standard errors for values <0.00001 were $<1.0 \times 10^{-6}$.
[c] The plasmid isolate pHB27RΔITN3-4 #5 also contained a spontaneous A to G point mutation at 38617, at the 58th position in the 77 nt long RNA molecule.

SOEing technique (Horton, 1993), as described in section 2.1.3.2. The new sequence was formulated so that no stem-loop structures, other than the terminating stem-loop structure would be able to form.

The new plasmid construct, pHB27R-R45OOP, was tested in the IP plaque assay for its ability to inhibit infecting phage development, Table 3.17. I expected that the *rep*P22 phage, λ*cI*857(*18,12*)P22, would be able to plate at high efficiency on 594[pHB27R-R45OOP] cells, and this was confirmed. However, the *rep*λ phage, λ*cI*857, also plated at relatively high efficiency on the 594[pHB27R-R45OOP] cells. These results were similar to results that were observed when λ*cI*857 infected cells containing the ori^{+} plasmids pHB28 and pHB27Rp_O^{-}, Table 3.14, where there was a near total suppression of the Inhibition Phenotype, when compared with the inhibition exhibited by cells with plasmid pHB27R. This result shows that OOP RNA, not merely the transcription of a 77 nt transcript from p_O, has an essential role to play in the IP.

3.2.7.2. pHB27RΔAT

The influence of the AT-rich region of *ori*λ from pHB27R on the IP was investigated by the deletion of this part of the plasmid. A detailed description of pHB27RΔAT construction can be found in section 2.1.3.2. This deletion should prevent active DNA replication initiation from *ori*λ, *i.e.* examine whether the inhibition of *rep*λ phages is caused by over-competitive replication of the plasmid triggered by the production of protein products of genes *O* and *P* by the infecting phage. Plasmid pHB27RΔAT retains the 4 iterons, and thus also provides a sink for

binding O protein, allowing an estimation of whether the IP is simply caused by competition between the infecting phage and plasmid copies for O protein binding. In short, this construct will be able to tell us if a functional origin is essential for the IP, or if the 4 iterons' ability to act as a binding site is enough to see the IP.

The new plasmid construct, pHB27RΔAT, was tested in the IP plaque assay for its ability to inhibit infecting phage development, Table 3.17. As I expected, the *rep*P22 phage, λ*cI*857(*18,12*)P22, was able to plate at high efficiency on 594[pHB27RΔAT] cells. The *rep*λ phage, λ*cI*857, was unable to plate on these cells, indicating that the plasmid construct pHB27RΔAT retained the IP. These results indicate that a functional origin is not necessary for the IP and that competitive replication initiation from the inhibiting plasmid is not required for the IP.

3.2.7.3. pHB27RΔITN(1-4)

The results with pHB27RΔAT demonstrated that in the presence of the four iterons, the IP was retained, so I decided to directly test if they were required for the IP. I decided to delete all four iterons of *ori*λ from pHB27R. pHB27RΔITN1-4 was constructed as described in section 2.1.3.2. This plasmid construct contains the t_O-*oop*-p_O element as well as the AT-rich region of *ori*λ. If the presence of the iterons is essential for the IP, then this ΔITN1-4 plasmid construct should concomitantly be defective for the IP. Both *rep*P22 and *rep*λ phages were able to plate at high efficiency on 594 host cells with plasmid pHB27RΔITN1-4, Table 3.17, indicating that the iterons are an essential component of *ori*λ required for the IP.

3.2.7.4. pHB27RΔITN(3-4)

It was next decided to delete two of the iterons from pHB27R, in order to determine if all four iterons were essential for the IP, or if the remaining two iterons would be sufficient to produce the IP. pHB27RΔITN3-4 was constructed using the SOEing technique (Horton, 1993), as described in section 2.1.3.2.

Several single colonies of the new 594[pHB27RΔITN3-4] constructs were tested in the IP plaque assay, Table 3.17. All tested single colonies allowed the *rep*P22 phage to plate at high efficiency. It became apparent that the new constructs produced one of three possible phenotypes when λ*cI*857 was plated on them. 594[pHB27RΔITN3-4] s.c.1 prevented the *rep*λ phage λ*cI*857 from plating (data not shown), retaining the IP, similar to wt pHB27R. Subsequent sequencing of this derivative revealed that the construct still contained all four iterons, indicating that the PCR mutagenesis technique was not successful in this isolate. 594[pHB27RΔITN3-4] s.c.2 allowed the *rep*λ phage λ*cI*857 to plate with relatively high efficiency, demonstrating the loss of the IP. Sequencing of this isolate revealed that iterons 3-4 had been successfully removed, as expected. These results indicated that loss of iterons 3 and 4 of *ori*λ from pHB27R prevented the IP, thus either iterons 3-4 are specifically required for the IP, or all four of the iterons are essential. 594[pHB27RΔITN3-4] s.c.5 allowed λ*cI*857 to plate with an efficiency close to 1.00 (*i.e.* higher than s.c.2). Sequencing of this isolate revealed that iterons 3-4 had been successfully deleted, as in s.c.2, but in addition to this deletion, s.c.5 also contained a point mutation within the nucleotide sequence of the *oop* gene, at 38617. This mutation caused an A to G change at position 58 of the 77 nt OOP RNA molecule.

The secondary A to G mutation within *oop* enhanced the loss of the IP in the 594[pHB27RΔITN3-4] construct. All subsequent assays utilized the 594[pHB27RΔITN3-4] s.c.2 isolate. No further characterization of the secondary *oop* gene mutation present in pHB27RΔITN3-4 s.c.5 has been done.

3.2.7.5. Testing OOP Phenotypes of New Plasmid Constructs

Before being used in any IP assay, all new plasmid constructs were tested for their ability to express OOP RNA. Because OOP RNA is known to inhibit CII expression, any plasmid over-expressing OOP RNA will prevent infecting phage from entering the lysogenic cycle, and will cause clear plaques to develop at 30°C. The *rep*P22 phage, λ*cI*857(*18,12*)P22, insensitive to the IP, was plated on host cells containing the plasmids to be tested, and plaque morphology was assayed, Table 3.18. At 30°C, λ*cI*857(*18,12*)P22 produced turbid plaques on 594 host cells, as expected. Clear plaques were seen on 594[pHB27R] cells at 30°C, demonstrating an OOP^+ phenotype. Turbid plaques were seen on 594[pHB27Rp_O^-] and 594[pHB27R-R45OOP] cells, indicating that the plasmids were no longer expressing OOP RNA (*i.e.* they are phenotypically OOP^-). Clear plaques were seen on 594[pHB27RΔAT], 594[pHB27RΔITN1-4] and 594[pHB27RΔITN3-4] cells, demonstrating that all of these plasmids remained OOP^+.

3.2.7.6. Effect of Plasmids Containing OOP-ori λ DNA on Survival Frequencies After *rep*λ Phage Infection

During strain construction, it was inadvertently noticed that cells in which

Table 3.18. Testing the OOP Phenotypes of New IP Plasmid Constructs

Host Strain	λ*cI*857(*18,12*)P22 Titer (pfu/mL) 30°C [a]	Plaque Morphology [b]	OOP Phenotype
594	1.7×10^{10}	Turbid	OOP^-
594[pHB27R]	2.1×10^{10}	Clear	OOP^+
594[pHB27Rp_O^-]	2.1×10^{10}	Turbid	OOP^-
594[pHB27R-R45OOP]	2.0×10^{10}	Turbid	OOP^-
594[pHB27RΔAT]	1.9×10^{10}	Clear	OOP^+
594[pHB27RΔITN1-4]	2.0×10^{10}	Clear	OOP^+
594[pHB27RΔITN3-4] [c]	1.8×10^{10}	Clear	OOP^+

[a] A 0.3 mL aliquot of fresh overnight stationary phase cells were mixed with 0.1 mL of phage and 3 mL of molten TB top agar and poured onto a TB plate. Plates were incubated overnight at 30°C and resultant pfu were analyzed.
[b] λcI857(18,12)P22 produces turbid plaques at 30°C. In cells over-expressing OOP RNA from a plasmid, clear plaques are seen at 30°C, due to the inhibition of lysogenization via OOP antisense regulation of gene *cII*.
[c] As previously stated in Section 3.2.7.4, all pHB27RΔITN3-4 assays utilized s.c.2.

OOP RNA was expressed from a plasmid were incapable of being lysogenized by an infecting phage (data not shown). It was decided to examine this observation in further detail by measuring the ability of cellular OOP RNA to shunt infecting λ phages toward the lytic mode of development.

594 cells with different OOP-*ori*λ plasmids were infected at 30°C with λ*cI*857 at an moi of approximately 2. The number of colonies surviving infection was counted and the percent cell viability was measured as the cell titer after infection (X 100) / cell titer at time 0, Table 3.19. Approximately 19% of 594 cells survived the infection withλ*cI*857. 10% of host cells expressing the IP, *i.e.* strains 594[pHB27R] and 594[pHB27RΔAT], survived the phage infection. Cells containing plasmids that were phenotypically OOP^-ori^+, *i.e.* strains 594[pHB27Rp_O^-], 594[pHB7R-R45OOP] and 594[pHB28], were increased for survival (26-51%) relative to the host cells without plasmids. Cells phenotypically OOP^+ori^-, *i.e.* strains 594[pHB29], 594[pHB27RΔITN1-4] and 594[pHB27RΔITN3-4], were decreased for survival (1-4%) compared to 594 host cells.

3.2.7.7. Testing Spacing Effects Between p_O and *ori*λ

Previous results in the Hayes lab (Bull, 1995) indicated that the spatial arrangement of t_O-*oop*-p_O to *ori*λ might play an essential role in the IP. Several derivatives of pHB50 (ΔiceOOP$^+$ori$^+$) had been constructed in which the distances between p_O and ITN-1 were altered (Bull, 1995). See section 2.1.3.1.2 and Fig. 3.19 for details. Briefly, pHB50 contains wt spacing between p_O and ITN-1; pHB51 contains a 60 bp deletion between p_O and ITN-1; pHB51kan contains a 1390 bp

Table 3.19. Influence of IP Plasmids on Host Cell Survival After Infection with the *rep*λ Phage λ*cI*857 at an moi of 2 at 30°C

Host Strain	Inhibition Phenotype [a]	Cell Viability [b] (+/- Standard Error)
594	No	0.19 +/- 0.02 [c]
594[pHB27R](OOP$^+$ori$^+$)	Yes	0.10 +/- 0
594[pHB27Rp_O^-] (OOP$^+$p$_O^-$ori$^+$)	Slight	0.51 +/- 0.02
594[pHB28](ori$^+$)	Slight	0.26 +/- 0.07
594[pHB29](OOP$^+$)	No	0.002 +/- 0.001
594[pHB27R-R45OOP] (OOP$^-$ori$^+$)	Slight	0.37 +/- 0.01
594[pHB27RΔAT] (OOP$^+$ori$^-$)	Yes	0.10 +/- 0.01
594[pHB27RΔITN1-4] (OOP$^+$ori$^-$)	No	0.002 [d]
594[pHB27RΔITN3-4] (OOP$^+$ori$^-$)	Slight	0.04 [d]

[a] Inhibition phenotype indicates whether the indicated host strain exhibits the IP. No = λ*cI*857 plates with high efficiency; Yes = λ*cI*857 is unable to plate; Slight = λ*cI*857 plates with low efficiency.
[b] Host cells were infected with λcI857 at 30°C at an moi of 2 for 75 minutes. Cell viability was calculated at the cell titer after λ*cI*857 infection / cell titer before infection.
[c] Values represent the average of two independent trials +/- standard error.
[d] 594[pHB27RΔITN1-4] and 594[pHB27RΔITN3-4] were each only assayed once.

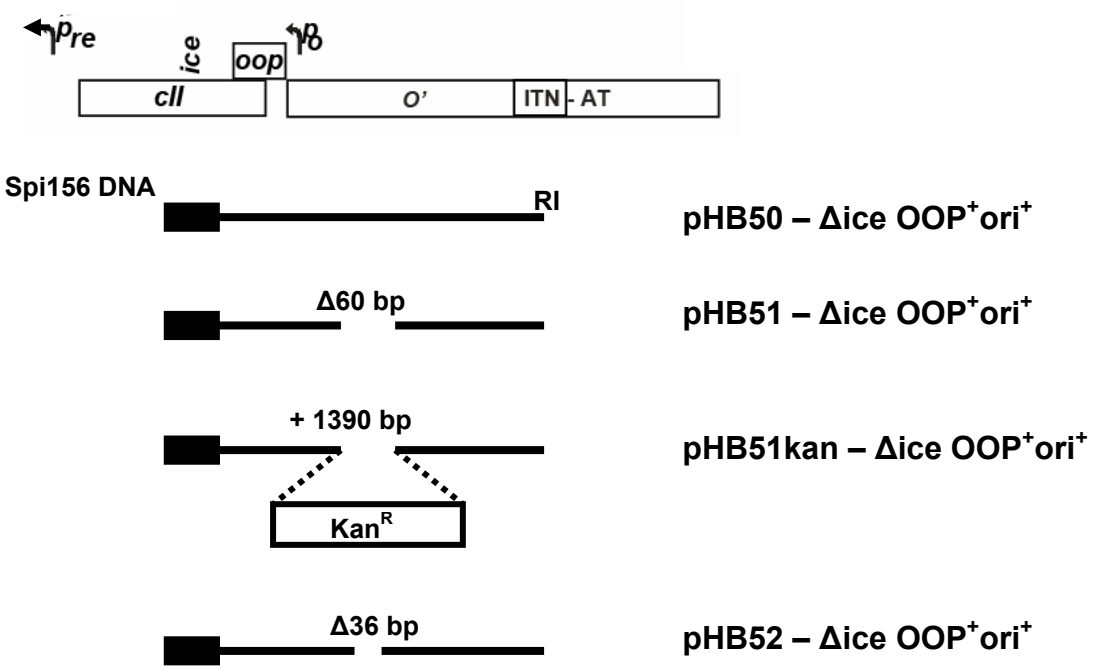

Figure 3.19. Inhibition Phenotype Plasmid Constructs Derived From pHB50. pHB51 contains a 60 bp deletion between p_O and *ori*λ. pHB51kan contains a kan^R marker cassette inserted between p_O and *ori*λ, creating a net increase of 1390 bp. pHB52 contains a partial deletion of the kan^R cassette, producing a net deletion of 36 bp between p_O and *ori*λ. All plasmid constructs were created by Harold Bull (1995).

insertion between p_O and ITN-1; and pHB52 contains a 36 bp deletion between p_O and ITN-1, Fig. 3.19. The previous study had shown that the 1390 bp insertion prevented the IP (λ*cI*857 plated with high efficiency) and the 36 bp deletion slightly interfered with the IP (λ*cI*857 plated with a low efficiency). Interestingly, the 60 bp deletion, which removed exactly 6 helical turns of DNA between p_O and ITN-1, had no effect on the IP (λ*cI*857 remained unable to plate). These results suggested a model where a possible interaction could be envisioned between the transcription complex bound at p_O and the replication complex bound at *ori*λ (Bull, 1995). This model proposed that transcription from p_O away from *ori*λ was essential to the IP, and that OOP RNA did not necessarily play a specific role in itself.

This presumption is in direct disagreement with the IP data presented for the plasmid construct pHB27R-R45OOP. This construct, while phenotypically OOP^-, should still transcribe a 77 nt transcript from p_O, away from *ori*λ. The loss of IP from this construct argues for the importance of OOP RNA itself, and against the argument for the importance of p_O transcription alone.

I decided to repeat the IP plaque assay using the various pHB50 derivatives constructed by Harold Bull (1995) in order to verify the previous findings that the spacing between p_O and *ori*λ can influence the IP.

Miniprep DNA was isolated from W3350[pHB50], W3350[pHB51] W3350[pHB51kan] and W3350[pHB52] frozen cultures. The plasmid DNA was then digested with *Eco*RI and *Eco*RV in order to visualize the λ fragments. pHB50 was expected to produce a fragment of 683 nt, pHB51 a fragment of 623 nt, pHB51kan a fragment of 2073 nt and pHB52 a fragment of 659 nt (Bull, 1995). As can be seen in

Table 3.20, all plasmid constructs appeared to contain fragments of reported sizes. pHB51kan was also confirmed the ability to infer Kan^R on host cells (data not shown).

The IP plaque assay was repeated, using the pHB50 derivatives. Infecting phages were λ*cI*857 (*rep*λ) and λ*cI*857(*18,12*)P22 (*rep*P22), Table 3.21. The assay was originally carried out in host strain W3350. All four plasmid derivatives retained their ability to inhibit *rep*λ phage development, regardless of spacing differences between p_O and ITN-1, contrary to previous results (Bull, 1995). The plasmids were transformed into the host strain 594, and the IP assay repeated, with the same results, Table 3.21. Changing spacing intervals between p_O and ITN-1 had no effect on the IP.

3.2.7.8. Influence of the SOS Response on the IP

It has been reported that *oop* is an SOS inducible gene, regulated by LexA (Lewis *et al,* 1994). However, data indicated that p_O transcription was only slightly elevated upon SOS induction (Lewis *et al*, 1994). I decided to see if a host cell containing a non-inducible LexA protein would still exhibit the IP. The IP plaque assay was repeated, using host strains 594 *lexA*3(Ind^-) and 594*lexA*3(Ind^-)[pHB27R] (OOP^+ori^+), Table 3.22.

The *rep*P22 phage, λ*cI*857(*18,12*)P22, was able to plate at high efficiency on 594*lexA*3(Ind^-)[pHB27R] (OOP^+ori^+) cells, Table 3.22, indicating that the *lexA*3 mutation did not influence lambdoid phage development. The *rep*λ phage, λ*cI*857, was unable to plate on 594*lexA*3(Ind^-)[pHB27R] (OOP^+ori^+) cells, Table 3.22. These results showed that the *lexA*3 allele did not influence the ability of an IP (OOP^+ori^+) plasmid to inhibit *rep*λ phage plating. This result suggested that OOP RNA

Table 3.20. *Eco*RI and *Eco*RV Double Digest of pHB50 Derivatives as an Assay to Measure λ DNA Fragment Sizes

Plasmid	Expected Band Size (nt)	Actual Band Size (nt) +/- Standard Error
pHB50 (wt)	683	687.9 +/- 4.0 [a]
pHB51 (Δ60)	623	613.1 +/- 3.6
pHB51kan (+1390)	2073	1941.8 +/- 58.2
pHB52 (Δ24)	659	668.4 +/- 7.7

[a] Plasmid DNA was digested with *Eco*RI and *Eco*RV as directed. Restricted DNA was visualized by running on a 1.2% agarose gel along with a low DNA mass ladder (from Invitrogen) as a marker. The markers were used to generate a standard curve, which was then used to estimate band sizes. Presented values represent the averages of two independent experiments.

Table 3.21. IP Plaque Assay for pHB50 Derivatives to Test the Influence of Spacing Alterations Between p_O and *ori*λ on the IP

Host Cells	Infecting Phage 30°C EOP +/- Standard Error [a]	
	λ*cI*857 [*rep*λ]	**λ*cI*857(*18,12*)P22 [*rep*P22]**
W3350	1.00 +/- 0 [b]	1.00 +/- 0
W3350[pHB50] (wt)	<0.00001	1.25 +/- 0.10
W3350[pHB51] (Δ60)	<0.00001	0.97 +/- 0.07
W3350[pHB51kan] (+1390)	<0.00001	1.29 +/- 0.28
W3350[pHB52] (Δ24)	<0.00001	1.10 +/- 0.09
594	1.00 [c]	1.00
594[pHB50] (wt)	<0.00001	0.81
594[pHB51] (Δ60)	<0.00001	1.13
594[pHB51kan] (+1390)	<0.00001	1.02
594[pHB52] (Δ24)	<0.00001	0.89

[a] Efficiency of plating is defined by phage titer on strain W3350[indicated plasmid] / titer on W3350 or titer on strain 594[indicated plasmid] / titer on 594.
A 0.3 mL aliquot of fresh overnight stationary phase cells and 0.1 mL of infecting phage and 3 mL of molten TB top agar were poured onto a TB plate and incubated overnight at 30°C. Resulting plaques were counted and the EOP was calculated.
[b] W3350 results represent the average of at least two individual trials. Standard errors for values <0.00001 were $<1.0 \times 10^{-6}$.
[c] 594 strains were only assayed once, thus no error values were available.

Table 3.22. Influence of a *lexA*3(Ind$^-$) Mutation on the IP Plaque Assay

Host Strain	λ*cI*857 30°C EOP [a]	λ*cI*857(*18,12*)P22 30°C EOP	Inhibition Phenotype
594*lexA*3	1.00 [b]	1.00	No
594*lexA*3[pHB27R] (OOP$^+$ori$^+$)	<0.00001	1.11	Yes

[a] A 0.3 mL aliquot of fresh overnight stationary phase cells were mixed with 0.1 mL of test phage and 3 mL of molten top agar and poured onto TB plates. Plates were incubated overnight at 30°C and resulting pfu were counted.
[b] Results are presented as efficiency of plating (EOP), defined as phage titer on 594*lexA*3[pHB27R] / titer on 594*lexA*3.

transcription from p_O was not being repressed by the non-inducible LexA3 protein, or that enough OOP RNA was still being produced to function in the IP.

Both infecting phages produced smaller plaques on 594 host cells containing the *lexA*3 allele (data not shown). In Fig. 3.14 it was demonstrated that phage infecting host cells which produced OOP RNA from the plasmid pHB29 produced larger plaques than when infecting host cells not producing OOP RNA. This suggests that lots of OOP RNA production results in large plaques. Here, the observed decrease in infecting phage plaque size in *lexA*3 host cells suggests that OOP RNA levels may be diminished by the *lexA*3 mutation.

3.2.8. SIP Phages

I decided to isolate spontaneous *rep*λ phage mutants resistant to the IP with the expectation that these mutations would fall within the phage target(s) of the IP.

3.2.8.1. Isolation of SIP Phages

The *rep*λ phage, λ*cI*857, previously shown to be sensitive to the IP, was plated on 594[pHB27R] (OOP^+ori^+) host cells at low dilution at 30°C. Any rare visible pfu, occurring at a frequency of about 3×10^{-6}, were picked, resuspended in buffer, and streaked on 594[pHB27R] cells. Any pfu that appeared relatively larger than the majority, were picked and resuspended in buffer. These purification steps were repeated a total of 13 times to produce ten independent λ*cI*857 SIP lysates (**S**uppress **I**nhibition **P**henotype) able to plate on 594[pHB27R] at high efficiency.

3.2.8.2. Characterization of SIP Phages

All ten SIP isolates plated on 594[pHB27R] cells at a much higher efficiency than the parental phage λ*cI*857 (*i.e.* an EOP of at least 0.32 for SIP phages compared to <0.00001for λ*cI*857) at 30°C and at 42°C. Several of the SIP isolates even grew better on 594[pHB27R] cells than on 594, Table 3.23. How this could be possible remains to be understood. SIP isolates 3 and 4 produced turbid plaques at 42°C, suggesting that they have acquired mutations affecting lysogenization. While the parental phage lysate for λ*cI*857 contained plaques of uniform size, all ten SIP phage lysates contained plaques of various sizes.

I decided to characterize the SIP phages in greater detail, in order to further understand the mechanism of the IP, specifically to understand the phage target of the IP.

3.2.8.2.1. SIP Phage Sequencing Data From o_R/p_R to *ori*λ

All ten SIP isolates were amplified by PCR using primers L22 (λ38517-38534) and R9+1 (λ39191-39175) and sent to NRC-PBI for sequencing from *ice* through *ori*λ. None of the ten SIP isolates contained any mutations within the t_O-*oop*-p_O or *ori*λ elements, as hypothesized. Three SIP isolates did contain point mutations within the N-terminal portion of gene *O*, Fig. 3.20. SIP-1 contained a point mutation at position 38817, with no resulting amino acid change. SIP isolates 2 and 7, independently derived, had the same point mutation at position 38822, causing a threonine to asparagine substitution.

I decided to sequence upstream from *ice* (the o_R/p_R region, the *cro* gene and the

Table 3.23. Plating Efficiencies and Plaque Morphology of SIP Phages

Infecting Phage	30°C		42°C	
	Phage EOP on 594[pHB27R] [a]	Plaque Morphology	Phage EOP on 594[pHB27R]	Plaque Morphology
λcI857	<0.00001	Turbid	<0.00001	Clear
SIP-1	0.50 +/- 0.03	Turbid	0.89 +/- 0.25	Clear
SIP-2	0.44 +/- 0.04	Turbid	0.86 +/- 0.14	Clear
SIP-3	1.12 +/- 0.04	Turbid	0.83 +/- 0.14	Turbid
SIP-4	2.94 +/- 0.28	Turbid	0.66 +/- 0.11	Turbid
SIP-5	1.71 +/- 0.51	Turbid	0.32 +/- 0	N/A [b]
SIP-6	0.79 +/- 0.30	Turbid	0.55 +/- 0.06	N/A
SIP-7	56.5 +/- 23.5	Turbid	0.93 +/- 0.14	N/A
SIP-8	13.0 +/- 7.55	Turbid	0.54 +/- 0.05	N/A
SIP-9	2.44 +/- 1.90	Turbid	0.58 +/- 0.06	N/A
SIP-10	4.61 +/- 1.78	Turbid	5.82 +/- 5.65	N/A

[a] Efficiency of plating is defined by phage titer on strain 594[pHB27R] / titer on 594. A 0.3 mL aliquot of fresh overnight stationary phase cells and 0.1 mL of infecting phage and 3 mL of molten TB top agar were poured onto a TB plate and incubated overnight at 30°C or 42°C Resulting plaques were counted and eop calculated. Presented results are the average of 2 independent experiments.
[b] N/A indicates that plaque morphology was not recorded.

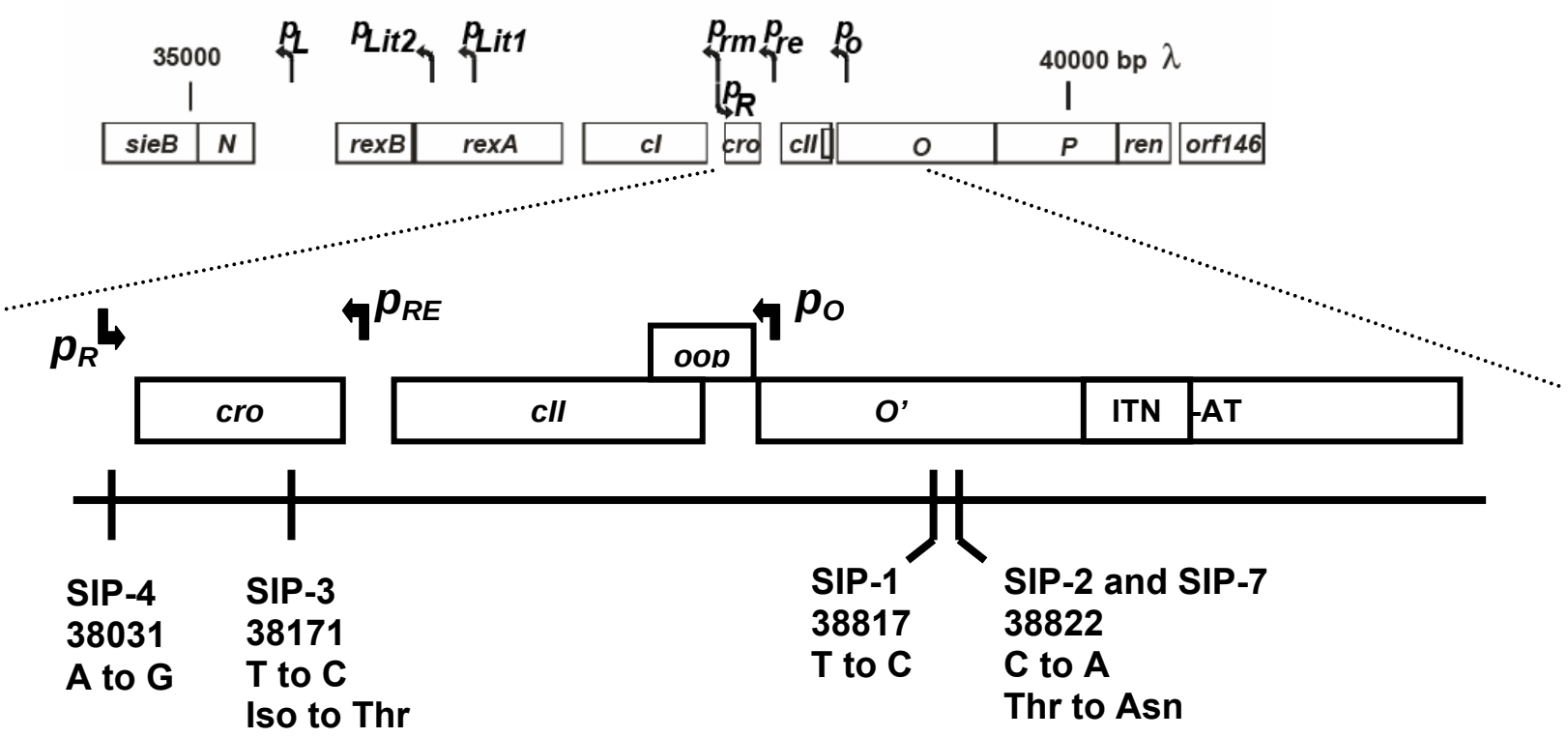

Figure 3.20. Relative Positions of SIP Phage Mutations. SIP phage isolates 1-10 were sequenced from *ice* through *ori*λ, using primers L22 and R9+1. Only phages 1, 2 and 7 showed any sequence variation in this region, specifically in the N-terminal region of gene *O*; SIP-1 at 38817 and SIP-2 and SIP-7 at 38822. SIP phage isolates 1-4 were also sequenced from p_R through *ice* using primers LMH29 and RPG6. SIP-4 contained a point mutation at 38031, within the SD site of the *cro* gene; and SIP-3 contained a point mutation at 38171, within the coding sequence of *cro*.

N-terminal portion of the *cII* gene) in SIP isolates 1-4. Primers LMH29 (λ37905-37922) and RPG6 (λ38569-38552) were used to amplify the isolates from the o_R/p_R region through *ice*. SIP isolates 1 and 2 (previously shown to carry point mutations within gene *O*) did not contain any sequence alterations in this interval. SIP-3 contained a point mutation at position 38171 creating an amino acid change (isoleucine to threonine) within the *cro* gene, Fig. 3.20. SIP-4 contained a point mutation at 38031, 8 nt downstream of the p_R transcriptional start site, within the SD sequence for the *cro* gene, Fig. 3.20.

3.2.8.2.2. Testing SIP Phages for pHB27R Integration

An alternative hypothesis for explaining SIP phage resistance to the IP was that they had incorporated the pHB27R plasmid into their genome via homologous recombination during the serial enrichment isolation protocol. Because the pHB27R plasmid contains λ DNA from 38517 to 39175 (from *ice* in gene *cII* through *ori*λ in gene *O*), the proposal that the plasmid could recombine into the phage genome is plausible. pHB27R is approximately 2800 bp long, and a λ phage head would still becapable of packaging a genome with such an insert (51302 bases). The λ genome is 48502 nt long, and a λ phage head is reported as being capable of packaging up to 106% of its genome (Maniatis *et al*, 1982), *i.e.* up to 51412 bases. A phage containing a pHB27R plasmid within its genome would possibly contain three functional origins of replication, its own *ori*λ, as well as an *ori*λ and a ColE1origin from the plasmid.

3.2.8.2.2.1. PCR Assay for pHB27R Integration

λ*cI*857 and SIP isolates 1-4 were amplified using primers LMH29 (λ37905-37922 and RMH25 (λ39626-39609**)** in order to amplify the phage genomes from the N-terminal region of *cI* through the N-terminal region of *P*. These two primers bind outside the region of sequence homology between pHB27R and the λ genome. In the absence of a pHB27R plasmid insertion, the phage PCR fragment was expected to be 1721 nt long. A SIP phage containing an inserted pHB27R plasmid would produce a PCR fragment of 4521 nt. The PCR products were visualized by running on an agarose gel, Fig. 3.21.

All amplified phages, λ*cI*857 and SIP isolates 1-4, produced PCR fragments of approximately 1721 bp, suggesting that the pHB27R plasmid has not been integrated within the SIP phage genomes (at least not between phage genes *cI* and *P*). The possibility still remains that the SIP phage lysates (which produce plaques of variable sizes) contain two types of phage, one containing the plasmid and the other not. The one without the plasmid would give the observed result.

3.2.8.2.2.2. Genetic Assays for pHB27R Integration

I decided to test for the presence of the pHB27R plasmid within the SIP genomes by genetic analysis. The plasmid pHB27R was derived from pBR322 and retains the ability to replicate from its ColE1 origin, *i.e.* independently of the λ origin. I decided to test the SIP phages' ability to replicate and produce a plaque when replication initiation from their λ origin was blocked. If the SIP phages were able to replicate under such conditions, it would strengthen the hypothesis that they were replicating from a pHB27R derived ColE1 origin or that the presence of two *ori*λ

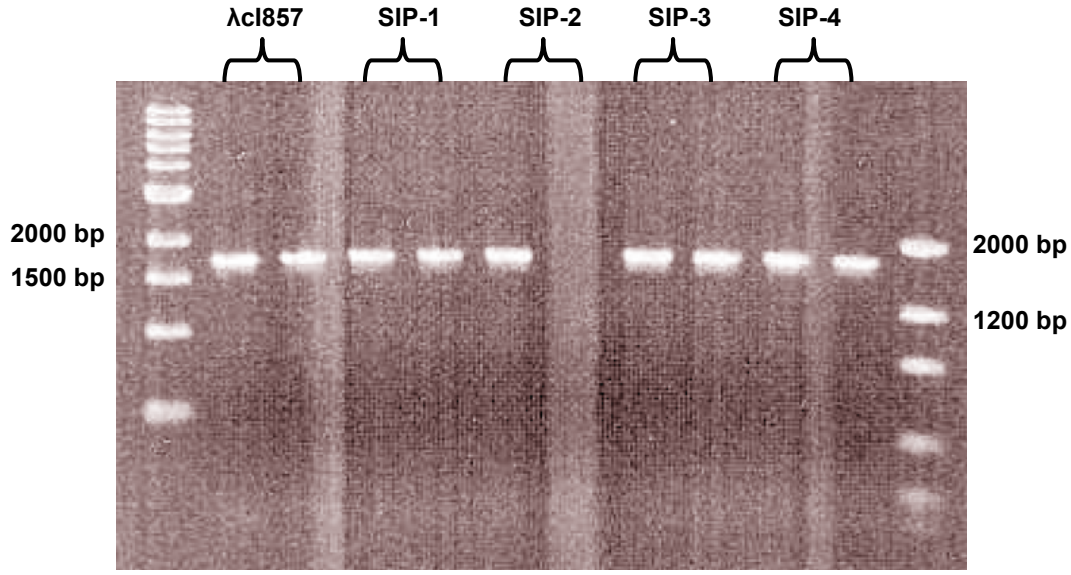

Figure 3.21. PCR Amplification of λ*cI*857 and SIP Phage Isolates 1-4, from Gene *cI* Through Gene *P*. The first lane contains a 1 Kb ladder, while the last lane contains a low DNA mass ladder, both from Invitrogen. The phages were amplified with primers LMH29 and RPG6. Each PCR was done in duplicate. λ*cI*857 produced the expected 1721 bp fragment. The SIP isolates also produced a 1721 bp fragment, indicating that the pHB27R plasmid has not been integrated into the SIP phage genomes between genes *cI* and *P*.

segments within a single phage genome allowed the phage to bypass *ori*λ-dependent replication initiation.

A r*ep*λ phage is unable to plate on 594 *dnaB*grpD55 host cells at 42°C because the *dnaB*grpD55 allele is non-functional for *ori*λ-dependent replication initiation, likely at the stage of P-DnaB interaction (Hayes *et al,* 2005). I decided to test the ability of the SIP isolates 1-4 to plate on 594 *dnaB*grpD55 cells at 42°C, Table 3.24. λ*cI*857 was unable to plate on 594 *dnaB*grpD55 cells at 42°C (EOP <0.00001), as expected. The SIP phage isolates were also unable to plate on 594 *dnaB*grpD55 cells at 42°C (EOP <0.00001). These results suggested that the SIP phages still require a functional DnaB protein in order to replicate, *i.e.* still undergo *ori*λ-dependent replication.

The plasmid pHB27R is *rop*$^{-}$, meaning ColE1 plasmid copy control number is poorly regulated in 594[pHB27R] cells, leading to a higher than normal plasmid copy number. Because plasmid pHB27 is *rop*$^{+}$, ColE1 plasmid copy number is more tightly regulated in 594[pHB27] cells. If SIP phages are replicating via a ColE1 origin, their replication should be diminished in 594[pHB27] host cells (as compared to levels in 594[pHB27R]). I compared SIP phage plating efficiencies on 594[pHB27] and 594[pHB27R] host cells at 30°C, Table 3.24. λ*cI*857 and SIP isolates 1-4 all plated with equal efficiencies on both host strains. These results indicated that the ColE1 Rop protein has no regulatory effect on SIP phage replication, suggesting that the isolates are not replicating from a ColE1 origin.

If the SIP isolates had integrated the pHB27R plasmid within their genome, the phages should now encode β-lactamase. 594 host cells were lysogenized with λ*cI*857

Table 3.24. Efficiency of Plating of SIP Phages Under Conditions Interfering with *ori*λ-dependent or *Col*E1-dependent DNA Replication Initiation

Infecting Phage	Host cells [a]			
	594	**594 *dnaB*grpD55**	**594[pHB27R]**	**594[pHB27]**
λ*cI*857	1.00 [b]	<0.00001 [c]	<0.00001	<0.00001
SIP-1	1.00	<0.00001	0.23	0.33
SIP-2	1.00	<0.00001	0.24	0.25
SIP-3	1.00	<0.00001	0.48	0.35
SIP-4	1.00	<0.00001	1.15	0.98

[a] A 0.3 mL aliquot of fresh overnight cells was mixed with 0.1 mL of infecting phage and 3 mL of molten TB agar and poured onto a TB plate and incubated overnight at 30°C or 42°C. Resulting plaques were counted and the EOP calculated.
[b] Results are presented as efficiency of plating (EOP), defined by phage titer on test strain / titer on 594.
[c] The 594 *dnaB*grpD55 assay was done at 42°C (the *grpD*55 mutation is ts). All other assays were carried out at 30°C.

and SIP phages 1-10 at 30°C. Lysogenic cells were streaked to TB and TB+Amp plates for single colonies. 594(λ*cI*857) cells grew on TB plates, but no colonies developed on TB+Amp plates, indicating that the 594(λ*cI*857) cells were not Amp^R. All ten 594(SIP) lysogen strains grew on TB plates as well as on TB+Amp plates. In all cases, more single colonies were seen on TB plates than on TB+Amp plates. These results demonstrated that all ten SIP isolates were Amp^R. The only logical explanation for this result is that the phage must have homologously recombined with the pHB27R plasmids during the selection process, and the amp^R gene from the integrated pHB27R is being expressed. Not every 594(SIP) single colony retained Amp^R. It is possible that the pHB27R plasmid recombines out of the phage genome at a high frequency. The plaque size variation seen in SIP lysates could be explained if phages which have lost their plasmid produced smaller plaques than those retaining the plasmid.

The Amp^R phenotype of SIP lysogens suggested that SIP phages contained an integrated pHB27R plasmid within their genome. The most likely explanation for this would be if the plasmid had recombined into the phage genome through the homologous sequence between genes *cII* and *O*. However, PCR amplification of this region did not support this hypothesis, Fig. 3.21. It remains possible that the plasmid has illegitimately recombined elsewhere in the phage genome. Alternatively, PCR amplified SIP plaques were grown up on 594 host cells. Perhaps in the absence of selection pressure, the plasmid was lost more readily and phages having lost their plasmids were the ones amplified via PCR. The PCR amplification should be repeated, using Amp^R 594(SIP) lysogens as sources of template DNA. The Amp^S 594(SIP) lyosgens should also be tested to determine whether they have lost the SIP

phenotype.

In summary, all ten SIP phages were able to plate at high efficiency on 594[pHB27R] (OOP^+ori^+) cells. None of the tested isolates were able to grow under conditions inhibiting *ori*λ-dependent replication initiation and none of the tested isolates were inhibited by the ColE1 Rop protein. These results suggested that the SIP isolates were not replicating from an integrated ColE1 origin. While none of the tested isolates contained an integrated pHB27R plasmid between phage genes *cI* and *P*, all ten isolates were able to produce Amp^R lysogens, suggesting that the plasmid pHB27R (or at least the amp^R gene) is integrated somewhere in the SIP phage genomes. Five of the SIP phages contained a single point mutation (all between p_R and ITN-1). Whether these mutations, shown in Fig. 3.20, are significant remains to be determined.

CHAPTER 4. DISCUSSION

4.1. P-INTERFERENCE

4.1.1. The Effect of λ *P* Expression on Host Cells – Conflicting Results in the Literature

Several independent researchers have studied the effect of λ *P* expression on host cells. Depending on the specific assay system utilized, results varied tremendously. The majority of the studies have utilized plasmids to express λ *P* with or without *O* co-expression. Most studies utilized hybrid plasmids, constructed by cloning the λ *imm* to *rep* region into a well characterized plasmid backbone, often pBR322. These plasmid constructs are capable of replicating from the pBR322-derived ColE1 origin, or from the λ-derived *ori*λ origin. pBR322 contains two selectable markers (Tet^R or Amp^R), thus the plasmids can be maintained and selected for very easily.

In 1978, Rao and Rogers studied two pBR322/λ hybrid plasmids, one containing a λ fragment consisting of genes *N* through *P* and one containing a λ fragment consisting of genes *N* through *cII*. This second construct will be discussed further in the section dealing with the IP. The first plasmid construct, containing λ genes *N* through *P* contained a temperature sensitive repressor gene and could be

induced for λ replication by shifting the cells to 42°C. This induced λ replication increased the plasmid copy number from approximately 50 at 30°C (due to ColE1 replication) to 130 at 42°C (Rao and Rogers, 1978). Cells carrying this plasmid construct were also killed at 42°C. The plating efficiency was decreased by 1000-fold at 42°C, and any survivor colonies were demonstrated to be ampicillin sensitive, *i.e.* had likely lost the plasmid. Rao and Rogers concluded that the killing was caused either by P-mediated titration of DnaB (as suggested to them by R. McMacken) or by some unknown gene to the right of *P* (unbeknownst to them, their construct contained gene *ren* immediately downstream of *P*). While Rao and Rogers may have demonstrated the first documented example of P-mediated cell killing, they did not attempt to characterize their finding any further.

In 1979, Klinkert and Klein cloned a fragment of λ DNA containing the C-terminal portion of gene *O*, and genes *P* through *Q*, into a high copy number plasmid RSF2124, under the control of a *lac* promoter. In this system, *P* gene expression could be induced by the addition of IPTG. The presence of the t_{R2} terminator downstream from *P* was expected to prevent expression of the *Q* gene. The O fragment was shown to be translated out of frame and was terminated after a five aa product was produced. Thus, the only detectable product upon the induction of the *lac* promoter was believed to be *P*. However, as in the Rao and Roger construct above, the researchers were as yet unaware of a gene situated immediately downstream of gene *P*, *i.e. ren*, which would also have been expressed. Klinkert and Klein demonstrated that when a high amount of P protein was produced by induction of the *lac* promoter with IPTG (*i.e.* 10-fold higher than in an induced λ lysogen), host cell

DNA synthesis was slowly inhibited, suggesting that P blocked the initiation step of *E. coli* DNA synthesis (Klinkert and Klein, 1979). *P* expression inhibited DNA synthesis in a manner indistinguishable from chloramphenicol treatment, which is known to inhibit the initiation step of DNA replication by blocking protein synthesis and thus preventing the formation of initiator protein(s) required for *E. coli* replication. They demonstrated that the induction of *P* expression resulted in an inhibition of host cell division. The average size of cells increased, filamentation occurred at a higher than normal rate and stationary phase was reached at a lower cell density when compared to cells in which *P* expression was not induced. Klinkert and Klein (1979) showed that *P* expression did not have a lethal effect on bacterial host cells. Although chromosomal DNA synthesis was inhibited, it did not appear to come to a complete stop. The culture cells were still able to divide, although at an extremely low frequency. The mechanism for P-mediated inhibition of host cell DNA synthesis was proposed by Klinkert and Klein (1979) to be due to the sequestering of the DnaB helicase by λ P.

In 1982, Tsurimoto *et al.* created a pBR322/λ plasmid construct that would produce O and P proteins at extremely high levels by placing the *O* and *P* genes under the control of tandemly repeated p_L and p_R promoters regulated by a temperature sensitive CI repressor. The tandem promoters were used to increase downstream gene expression. The plasmid construct was deleted for *cro* in order to increase p_R transcription, and deleted for the termination site t_{R1} to increase transcription of the *O* and *P* genes. The new construct produced O and P proteins at 42°C to the extent that they constituted several percent of the total *E. coli* cellular protein (Tsurimoto *et al,*

1982). It was realized, however, that host cells carrying this plasmid could not grow at 42°C; DNA replication was virtually arrested within 30 minutes and protein synthesis and cell viability gradually decreased. In contrast to the results seen by Klinkert and Klein (1979), filamentous cells were not seen. Tsurimoto *et al.* concluded that the loss of DNA synthesis and cell viability was due to the over-expression of the λ *P* gene. They suggested that any differences seen in their system and that of Klinkert and Klein was due to the simultaneous over-expression of *O* with *P,* although no possible mechanism was provided.

In 1991(a), Maiti *et al.* studied a pBR322/λ hybrid that constitutively expressed λ *P*. Their construct, pMR45, consisted of a λ DNA fragment containing the N-terminal portion of gene *cI*, O_R/p_R, gene *cro,* the t_{R1} terminator site, genes *cII, O, P, ren* and the t_{R2} terminator site. The plasmid contained a *cro* mutation which interfered with p_R regulation, causing constitutive p_R transcription and subsequent *cro, cII, O, P* and *ren* gene expression. It was demonstrated that the transcription of genes downstream of t_{R1} was increased by the *cro* mutation. They demonstrated that plasmid pMR45, constitutively expressing *P*, did not yield stable transformants of host cells. Deletion of gene *P (*and *ren*) removed the lethal effect, demonstrating that the lethality was P-mediated (Maiti *et al,* 1991a). Deletion of the N-terminal half of the *O* gene did not disrupt P-mediated host cell lethality, demonstrating that the killing effect exerted by P was λ replication-independent. Maiti et al. (1991a) tested whether the lethal action of the P protein was dependent on its well documented interaction with DnaB. Various GroP strains (Georgopolous and Herskowitz, 1971), containing *dnaB, dnaJ* or *dnaK* mutations preventing λ replication and suppressible by mutations in *P*, were

tested for their ability to survive transformation with pMR45. All tested GroP mutants (GroPA15, GroPB558 and GroPC259) were as susceptible to P-killing as wild type host cells. These results suggested that P-killing was not due to the sequestering of DnaB. However, results reported as "data not presented" showed that lethal levels of P protein inhibited host DNA synthesis significantly, suggesting that P might be involved in an inhibitory reaction with some other component of the host DNA synthesis machinery. Host mutations, named Rpl (**R**esistant to **P-**gene **l**ethality), were isolated which could survive transformation with pMR45 (Maiti *et al,* 1991b).

Datta *et al.* (2005a) characterized the Rpl mutants isolated by Maiti *et al.* in 1991(b). They demonstrated that P protein produced from pMR45 inhibited host cell DNA synthesis at the stage of replication initiation (Datta *et al,* 2005a). The rpl mutations mapped to the *dnaA* gene of *E. coli*, leading to the proposal that DnaA is the target of P-mediated lethality (Datta *et al,* 2005a). The *dnaA* Rpl mutants were resistant to P-killing, but remained active for *E. coli* DNA replication. *In vitro* assays, using purified proteins, demonstrated that P protein inhibited the binding of wild type DnaA to *ori*C DNA and ATP, inhibiting host DNA replication initiation, ultimately leading to cell death (Datta *et al,* 2005b); however, the P protein was not able to inhibit the *ori*C or ATP binding of the *dnaA* Rpl mutant. These results directly demonstrated that P-mediated cell killing, which inhibited cellular DNA replication initiation, was suppressed by mutations in *dnaA*. This group of workers did not assay cell morphology to determine whether high levels of P protein induced cellular filamentation. As well, it remains to be determined whether a DnaA-P interaction can interfere with P's activity in λ replication initiation.

The Mandal group (Maiti *et al,* 1991a; Maiti *et al,* 1991b; Datta *et al,* 2005a; Datta *et al,* 2005b) used a transformation assay to study P-killing, but the plasmid system they utilized had a major flaw that has not been accounted for. The pMR45 plasmid that they used constitutively expressed *P* at high levels and did not yield stable transformants of *E. coli* cells. In order to propagate the plasmid, they had to transform it into cells lysogenic for λ. The λ prophage produced CI protein, which repressed the p_R promoter of pMR45, inhibiting *P* expression. I was able to obtain some 594 (*i.e.* non-lysogenic host cells) transformants using pMR45 DNA received from their laboratory. According to their published results, this should not be possible. Personal communication with Dr. Mandal revealed that all pMR45 plasmid preparations used in their studies were likely contaminated with λ DNA. Dr. Mandal proposed that my survivor 594[pMR45] transformants had also obtained λ DNA and were thus lysogenic, *i.e.* were 594(λ)[pMR45]. Genetic testing of my transformants confirmed that they had somehow obtained λ immunity, even though the plasmid pMR45 does not encode the entire *cI* gene. I demonstrated that a 5 μl aliqout of pMR45 DNA, received from Dr. Mandal, did not produce any λ plaques; however it remains possible that the plasmid preparation may have contained a low titer of contaminating infectious phage particles. Dr. Mandal proposes that contaminating λ prophage DNA, co-isolated during the plasmid purification protocol, was transformed into cells at the same time as the pMR45 DNA. This does not seem very plausible, as the probability of a single cell obtaining two separate DNA molecules during a single transformation event would be extremely rare. A more feasible explanation is that the plasmid and the prophage DNA, homologous from the N-terminal of the *cI* gene

thorough t_{R2}, have undergone some sort of homologous recombination event while both elements were present in the lysogenic host cell. The nature of this molecule remains unknown, and what possible effect it could have on any experiments also remains speculative. For example, if an rpl isolate, able to survive transformation with pMR45, actually contained this recombinant plasmid/phage, the possible presence of the *cI* repressor gene would prevent *P* expression, and thus allow cell survival. The presence of a *dnaA* mutation would be completely irrelevant. Because of the problems that have been raised regarding the pMR45 contamination, the validity of the entire system is called into question.

In summary, it appears that λ P protein, over-expressed (compared to levels from a developing phage) from a plasmid construct, interferes with host cell DNA synthesis. Different assay systems produced varying results. Assays differed in the amount of P protein produced; and essentially, higher levels of *P* expression appear to be more toxic to host cells, as might be expected. An intriguing finding, however, is the effect of *O* gene co-expression on the severity of P-Interference. It appears that when *P* is co-expressed with *O*, P-killing is less severe. Most groups concluded that P-killing was likely due to the sequestering of DnaB and inhibition of its function in host cell DNA replication. One group had an alternative conclusion and suggested that P-killing was due to an inhibition of the DnaA protein. I wished to try and determine whether P kills host cells and whether P-mediated cellular filamentation occurs. I also wished to elucidate a mechanism for this P-effect.

4.1.2. The Effect of λ P, Expressed from Plasmid pHB30, on Host Cells

The system that I used for studying the P-effect used a series of plasmids constructed by Harold Bull (1995). Essentially, pHB30 is a pBR322/λ hybrid containing a λ DNA fragment containing the λ gene *cI[*ts]857, the o_R/p_R region, a *Cro-O* fusion gene, *P, ren* and the terminator site t_{R2}. *P* gene expression is regulated by a temperature sensitive repressor. This construct is deleted for the t_{R1} terminator site, likely increasing the transcription of *P* and *ren*. As the λ replicator element is not completely intact, the plasmids are only capable of replicating from the pRB322-encoded ColE1 origin. Plasmids pHB31, pHB33 and pHB35 contain various *P* gene deletions. These plasmids, similar to the construct used by Klinkert and Klein (1979), express *P* (or parts of *P*), in the absence of *O*. However, while Klinkert and Klein's *P* gene was regulated by a *lac* promoter; in pHB30, *P* is expressed from a p_R promoter. It has been demonstrated that p_R is a much stronger promoter than *plac* (Aris *et al,* 1998), especially in the absence of active Cro (Tsurimoto *et al,* 1982), as in our construct. Thus, it can be inferred that *P* expression levels in my system are higher than in the Klinkert and Klein system.

When I transformed pHB30 into host cells, in an "all or none" transformation assay similar to the one used by Maiti *et al.* (1991a), I found that at temperatures where *P* expression was high, no survivor transformant colonies developed. Plasmids deleted for *P* were able to be successfully transformed at elevated temperatures. These results suggested that P protein, expressed in high levels from pHB30 at 42°C, was lethal to host cells.

I assayed the effect of P protein on host cells using an assay where *P* expression could be transiently induced. I induced *P* gene expression from cells

containing pHB30 for up to five hours and monitored cell growth, cell morphology and cell survival. These results indicated that *P* expression from pHB30 inhibited cell division, likely as a consequence of inhibiting cellular DNA synthesis (Hiroto *et al,* 1968), resulting in the development of filaments. Host cells defective for the induction of the SOS response (594*lexA*3 cells) did not form filaments, suggesting that P was capable of triggering SOS-mediated cellular filamentation. The SOS response is induced as a consequence of a cellular response to the inhibition of DNA propagation as reviewed by Friedberg *et al.* (1995). In general, the evidence suggests that an induction of the SOS response involves the presence of ssDNA, to which RecA binds and is in turn activated. The activated RecA protein stimulates LexA autocleavage. Cleaved LexA repressor falls off of the promoters of SOS genes, allowing gene expression. The inhibition of DNA propagation leads to the appearance of ssDNA, an inducer of the SOS response. For example,

> "many temperature-sensitive *dna* mutants, in which the elongation phase of DNA replication is inhibited at the restrictive temperature, exhibit SOS induction when shifted to higher temperature" (Friedberg *et al,* 1995).

In contrast, temperature sensitive *dna* mutants that prevent replication initiation, *e.g. dnaA*[ts], theoretically should not induce the SOS response, because ongoing DNA propagation would proceed to normal termination without further rounds of DNA synthesis. The finding that high levels of P protein, expressed from pHB30 at 42°C, induced SOS-mediated cellular filamentation suggested that high levels of P protein interfered with the elongation stage of DNA replication. This observation is not in disagreement with the findings by Klinkert and Klein (1979) which suggested that P protein inhibited the initiation stage of DNA replication, which can be explained by P

out-competing DnaC for DnaB, and thus also serving to inhibit replication initiation. My results suggest that *P* over-expression can interfere with both the initiation and elongation stages of *E. coli* DNA replication, and that P-triggered cellular filamentation requires some expression of a gene(s) that is normally repressed by LexA.

It has been shown, using plasmid DNA templates, that high levels of protein expression downstream of cloned p_R and p_L promoters can induce the host's SOS response (Aris *et al,* 1998). I questioned whether the filamentation observed upon derepression of p_R transcription from pHB30 was actually due to a specific function of P, or merely a response to the high level of protein produced from the p_R promoter. Cells expressing *P* from pHB30 at 42°C exhibited the SOS-mediated filamentation phenotype, while cells expressing the in-frame partially deleted *P* from pHB31 did not. These findings suggested that functional P protein, rather than merely high protein expression, was responsible for the observed SOS-mediated cellular filamentation.

I demonstrated that 35% of cells survived a five hour exposure to high levels of *P* expression from pHB30. It was decided to amend the term P-killing to P-Interference, to emphasize the finding that *P* expression in itself is not lethal to host cells. P protein appears to inhibit host DNA replication, which will eventually be lethal if P expression is not turned off. The finding that cells can survive transient *P* expression led me to investigate cellular mechanisms for degrading accumulated P protein. I showed that host cells containing a defect in the ClpXP protease were nearly 100-fold more sensitive to P-mediated cell killing. This result suggested that

ClpXP is primarily responsible for degrading P protein in host cells.

Plasmids pHB31, pHB33 and pHB35, all P^-, did not exhibit P-Interference; however, they did interfere with host cell growth. It has been shown that the expression of proteins from the strong p_R and p_L promoters can depress host cell growth, due to the increased metabolic burden of excess protein production (Aris *et al,* 1998). The production of large amounts of protein from p_R and p_L is energetically draining and can interfere with cell metabolism. This can explain the observation that cells containing plasmids transcribing from p_R at 42°C have slower growth rates, even in the absence of P protein.

It had recently been demonstrated that host cells containing mutations in *dnaB* (GrpD55 and GrpA80) could be transformed with pHB30 at 42°C (Bull, 1995), suggesting that they were resistant to P-killing. The *grpD55* allele of *dnaB* has been shown to be functional for *E. coli* replication, yet is unable to support λ replication initiation, and could be suppressed by a mutation in gene *P* (Saito and Uchida, 1977). The easiest way to explain the *grpD55* allele is that the mutated DnaB protein is unable to bind to P, making it inaccessible for λ replication, and free to continue on with *E. coli* replication. When I transiently induced pHB30 for five hours in a 594 *dnaB*grpD55 strain, however, I found that these cells remained sensitive to P-Interference. These results can be interpreted in one of two ways. First, the P-Interference phenotype, in wild type and in *dnaB*grpD55 cells, is independent of DnaB-P interactions that serve to limit the availability of DnaB. This proposal appears unlikely, since P was demonstrated to bind DnaB; since DnaB is present in limiting amounts in *E. coli* (*i.e.* 20 hexamers per cell); and since P is able to out-

compete DnaC for binding to DnaB. It is unfathomable to think that high levels of P protein would not complex to the DnaB present in the cell, and that the formation of this complex would not restrict the ability of DnaB to participate in *E. coli* replication. A second way to interpret the inability of the *grpD55* allele of *dnaB* to escape P-Interference is that P still binds to the mutated $DnaB_{grpD55}$ protein, preventing it from functioning in *E. coli* replication, but that the P-$DnaB_{grpD55}$ complex is non-functional in λ replication. One such possibility is that the P-$DnaB_{grpD55}$ complex may not be dissociated by the heat shock proteins, thus $DnaB_{grpD55}$ would remain in a dead-end complex with P.

The finding by Dr. Mandal's group (Maiti *et al,* 1991b; Datta *et al,* 2005a; Datta *et al,* 2005b) that P-killing can be suppressed by a mutation in *dnaA* led to the hypothesis that DnaA is the cellular target in P-killing. We received a strain reported to carry the *dnaA* rpl8 defect to use in the P-Interference assay. While the cells demonstrated a slow growing phenotype, as reported, they remained sensitive to P-Interference. Subsequent sequencing analysis of the *dnaA* gene of the strain indicated that it did not contain an rpl mutation, leading to the suggestion that the mutation had spontaneously reverted to wild type, leaving the cells with another mutation(s) causing the slow-growing phenotype, which had been attributed to the *dnaA*rpl8 allele. Thus, I was unable to confirm or deny the finding that a *dnaA* mutation could suppress P-Interference from pHB30.

I demonstrated that P protein, expressed from the plasmid pHB30 at 42°C, interfered with replicative killing in *cis* from the host strain Y836. Y836 cells contain a cryptic λ prophage which can be induced to replicate by shifting the cells to 42°C.

Because the cryptic λ prophage does not contain the genes required to excise from the bacterial chromosome, the initiation of *ori*λ-dependent replication is lethal, *i.e. cis* replicative killing. High levels of P protein, made from pHB30, increased the number of 42°C cell survivors by 10-fold. This result suggested that high levels of P protein can also interfere with λ replication initiation.

I was able to isolate pHB30 plasmid isolates able to produce stable transformants at 42°C, which I called pHB30$^{nl\text{-}42}$. I determined that the majority of my isolated plasmids contained a point mutation which reverted the *cI*[ts]857 mutation back to *cI*$^{+}$ (a very rare event), thus preventing the expression of the *P* gene at all temperatures. This result suggested that the plasmid was under a strong selection pressure to inhibit *P* gene expression and that the simplest way of accomplishing this was to repress *P* gene expression. This suggested that a simple point mutation of the *P* gene was likely not able to inhibit P-interference.

4.1.3. Possible Roles for DnaA in λ Replication

The findings by Dr. Mandal's group regarding a potential interaction between P and DnaA led me to search for any other evidence for such an interaction and its possible relevance for λ replication. It has been known since the late 1970's that some *dnaA* mutations prevent the replication of λdv plasmids (Kellenberger-Gujer and Pokhajska, 1978) or pBR322/λ hybrid plasmids (Kur *et al,* 1987), although DnaA had been shown to be dispensable for phage λ replication initiation and DNA propagation (Hayes, 1979). Interestingly, this *dnaA* inhibition of plasmid replication could be suppressed by π or amber mutations in gene *P* (Kellenberger-Gujer and Pokhajska,

1978; Kur *et al,* 1987).

λdv plasmids, isolated from the λvir phage by Matsubara and Kaiser in 1968, contain only the genetic elements required to autonomously replicate themselves. It has been estimated that there are approximately 60 copies of λdv plasmids per cell (Matsubara and Kaiser, 1968). However, Hayes *et al.* (1997) have more recently estimated the λdv copy number to be closer to 25 copies per cell. In essence, λdv plasmids contain a λvir DNA fragment from gene *rexB* through gene *P* (Matsubara and Kaiser, 1968; Berg, 1971). As the λdv plasmid was derived from λvir, which contains three operator site mutations (v2v1v3), CI protein is unable to bind to λdv operator sites and inhibit p_R transcription, allowing for constitutive expression of the *cro, cII, O* and *P* genes. The presence of the O and P initiator proteins, *ori*λ (present within the coding sequence of gene O) and transcriptional activation from p_R, allows *ori*λ-dependent θ replication initiation to proceed. The Cro protein is believed to play an important regulatory role in λdv plasmid replication, regulating the amount of transcriptional activation from the p_R promoter and regulating the amount of *cII, O* and *P* gene expression. The λdv construct does not express the N antiterminator protein, which results in about 80% of p_R transcription terminating at t_{R1} (before genes *O* and *P*), which was proposed to keep the O and P proteins at relatively low levels (Rosenberg *et al,* 1978). However, it has not been determined exactly how the levels of O and P protein produced from a λdv plasmid compare to the levels made during a phage infection or prophage induction. Despite the active replication of λdv, its presence does not seem to harm host cells, as the growth rates of isogenic bacteria with or without λdv are similar (Matsubara and Kaiser, 1968).

4.1.3.1. Model I: DnaA Regulates Transcription From the p_R Promoter

Why DnaA is apparently required for λdv replication, yet is dispensable for phage λ replication remains uncertain. During phage λ replication, the N protein plays an important role in positively regulating *O* and *P* gene expression and likely in transcriptional activation of *ori*λ, by its function in allowing p_R transcription to continue through t_{R1} (Rosenberg *et al,* 1978). In contrast, λdv constructs are N^-, leading to less transcription through *O, P* and *ori*λ. DnaA is a transcriptional activator and has been shown to bind to and activate transcription from p_R (Wegrzyn *et al,* 1995). I propose that it is possible that DnaA is required for λdv replication, merely as an alternative method of ensuring that sufficient levels of transcription pass through t_{R1} to support *ori*λ replication in the absence of *N* gene function. It would be interesting to see if the addition of the *N* gene to λdv constructs would negate the requirement of *dnaA* for plasmid maintenance.

4.1.3.2. Model II: DnaA-P Interaction Helps Load DnaB Helicase onto *ori*λ DNA

The finding that mutations in *dnaA* preventing λdv plasmid replication can be suppressed by π mutations in gene *P* suggests that DnaA might have a more specific role in λdv plasmid and phage λ replication (Kellenberger-Gujer and Podhajska, 1978; Kur *et al,* 1987; Wegrzyn *et al,* 1996). The finding that P-mediated cell killing can be suppressed by a mutation in *dnaA* (Datta *et al,* 2005a) further supports a model where P and DnaA directly interact. It is very intriguing that a single point mutation in gene *P*, producing the π phenotype (πA66), can suppress mutations inhibiting λ replication in many different host genes, *i.e. dnaA, dnaB, dnaJ, dnaK* and *grpE*. It is known that

this π mutant has a decreased binding affinity for DnaB (Konieczny and Marszalek, 1995), leading to the hypothesis that the heat shock proteins are no longer required to dissociate the π-DnaB complex, thus making λ replication independent of the heat shock proteins.

There is evidence for P interacting with DnaA, P interacting with DnaB, and DnaB interacting with DnaA. Whether a P-DnaB-DnaA complex can develop is unknown. I suggest the possibility that P protein can bind to DnaB that is already present at *ori*C, during the stage that where is also bound to DnaA; *i.e.* P may also be able to dissociate pre-formed *ori*C-DnaA-DnaB complexes in a similar manner to the experimentally documented P-mediated dissociation of DnaB-DnaC complexes.

It has been suggested that DnaA is involved in the heat shock protein-mediated removal of P from DnaB, however this model has never been tested experimentally (Wegrzyn *et al,* 1996). Supposing this model is true, why should DnaA only be required in removing P from DnaB in the λdv system, but not phage λ replication? On the other hand, DnaA is proposed to negatively regulate the transcription of the *rpoH* gene, and could thereby regulate the expression of the heat shock proteins (Wang and Kaguni, 1989), which have been shown to be essential for the proper loading of DnaB onto *ori*λ DNA.

Many of the λdv studies show that plasmids containing mutant (π) *P* genes cannot replicate in wild type host strains, but can develop in *dnaA*46[ts] strains. It has been proposed that precise DnaA-regulated transcriptional activation of *ori*λ may be important for the proper loading of DnaB onto λ DNA by the weakly binding π protein (Szalewska-Palasz *et al,* 1998). These researchers have suggested that too much

transcriptional activation (by wild type DnaA) could destroy the delicate *ori*λ-O-πP-DnaB complex prematurely, preventing replication. A low level of transcriptional activation (in *dnaA*-defective hosts) also inhibits λdv replication, as the weak binding affinity of π for DnaB necessitates high levels of π expression, *i.e.* because π is a poor competitor for binding DnaB, a lot of π must be made to compensate. These researchers proposed that the DnaA46ts allele, which is not as active for p_R transcription as wild type DnaA, produces an optimal level of transcription from p_R for active replication of a λdv plasmid containing a π mutation in gene *P* (Szalewska-Palasz *et al,* 1998).

4.1.3.3. Model III: DnaA is Required for the Initiation of Bidirectional Replication from *ori*λ

It has been demonstrated that a region of DNA sequence near the start of the *O* gene (*i.e.* in the p_O region) strongly resembles the *ori*C binding site for DnaA (Taylor and Wegrzyn, 1995). This same region has also been implicated in DnaA activity. It has never been understood how the leftward replication fork could open in the presence of the "O-some". Perhaps DnaA binding to a DNA sequence to the left of the "O-some" can somehow mediate the unwinding of the DNA in this region, allowing the propagation of the leftward replication fork. Again, no experimental evidence exists to support this model.

4.1.3.4. Model IV: DnaA is Required to Regulate the Switch From the θ to σ Modes of λ Replication

Yet another model suggests that DnaA is important in regulating the switch from the θ to the σ mode of λ replication (Szalewska-Palasz *et al,* 1998; Konopa *et al,* 2000; Datta *et al,* 2005b). This model proposes that DnaA is initially required to activate transcription from p_R, which increases the levels of O and P proteins and ensures that *ori*λ is transcriptionally activated; thus DnaA initially enhances the θ mode of replication. After several rounds of θ replication, the number of λ genomes increases and DnaA begins to be titrated out; and P binds to DnaA, preventing it from transcriptionally activating the origin; both simultaneous events are proposed to eventually lead to the presence of phage genomes without DnaA-mediated p_R transcription. This is proposed to lead to a single round of unidirectional θ replication, followed by σ replication. The interaction between P and DnaA is proposed to inhibit θ replication in two ways. The P-DnaA interaction inhibits DnaA activity, preventing p_R transcription, leading to an inhibition of *O* and *P* expression and of *ori*λ transcriptional activation; and P bound to DnaA is unable to function in θ replication. Essentially, this model does not take into account the fact that DnaA is required for λdv replication, but not phage λ replication. Phage λ replication progresses from the θ to the σ mode of λ replication; while λdv plasmids apparently replicate solely via the θ mode of replication. Thus, this model proposes an explanation of how DnaA would be required for phage replication, not λdv replication, where it has been shown to be required.

However, the model can be simplified as follows. It is essentially proposed that high levels of DnaA promote bidirectional θ replication (Konopa *et al,* 2000). Once available DnaA levels decrease, replication switches to the σ mode. Thus, λdv

plasmid replication, which must rely solely on the θ mode of replication, requires high levels of DnaA. λdv plasmids cannot produce stable replication products via the σ mode because they do not encode the *gam* gene. Any DS concatemeric DNA produced will be degraded by the host ExoV enzyme. In *dnaA*-defective hosts, less transcriptional activation from the p_R promoter will lead to one round of unidirectional θ replication, followed by the shunt to σ replication (Konopa *et al,* 2000), a potentially disastrous event for plasmid stability and maintenance. This may be a way to explain the inability of λdv plasmids to be stably maintained in *dnaA*-defective host cells. It can be suggested that λdv plasmids replicate via rolling circle replication in *dnaA*-defective cells, but because the plasmids do not encode the *gam* gene, the linear DS DNA replication products are degraded by ExoV. Phage λ replication, on the other hand, is less dependent on DnaA, as only a few rounds of θ replication occur, followed by a switch to σ replication. The presence of *gam,* expressed from phage λ, allows the linear DS DNA products of rolling circle replication to stably exist in the cell.

In one specific situation, *dnaA* has been demonstrated to be essential for phage λ replication (Szalewska-Palasz *et al,* 1998; Konopa *et al,* 2000). Phages containing *P*ts1πA66 defects were unable to replicate in wild type *E. coli* cells, but were able to replicate in *dnaA*46[ts] host cells at 43°C. P^+ phage were able to replicate in $dnaA^+$ and *dnaA*46[ts] cells equally well. In *dnaA*46[ts] cells, the replication of the *Pt*s1πA66 phage occurred predominantly by the σ mode, as early as five minutes after infection. Replication of the P^+ phage in $dnaA^+$ cells occurred predominantly via the θ mode (Konopa *et al,* 2000). These results strengthen the hypothesis that DnaA is important in regulating the switch from the θ to σ modes of λ replication. Again, the

proposal is that high levels of DnaA support the θ mode of replication and low levels promote the switch to the σ mode of replication. λdv plasmids are more dependent on DnaA levels as the switch to the σ mode of replication would likely lead to loss of plasmid stability and maintenance (Silberstein and Cohen, 1987; Kusano *et al,* 1989; Silberstein *et al,* 1990). Any plasmid that replicates via the σ mode of replication and somehow escapes ExoV degradation of its DNA is still likely to have problems being stably maintained in growing host cells. The replication products are present in one long, linear structure, which is unable to be partitioned and segragated into daughter cells. The parental cell will retain the plasmid, with all daughter cells remaining plasmid-free.

These studies on the switch from the θ to σ modes of replication demonstrated no results to exclude the participation of recombination in generating σ structures. Any type of strand invasion event between two replicating DNA molecules will lead to concatameric DNA and σ structures (Kogoma, 1997; Motamedi *et al*, 1999). It is known that late replicating λ molecules form σ-like structures, and eventually long, linear DS concatemers. The mechanism by which these structures develop remains controversial. The process that has been dubbed rolling circle replication likely involves both recombination and DNA replication events. For example, the generation of a SS nick in a circular monomer would provide a free 3' end which would serve as a SS capable of invading the dsDNA of another circular monomer. Subsequent DNA synthesis would produce a long, linear recombinant molecule.

4.1.4. Effect of *P* Expression on Host Cells – Dependence on the Ability of P to Participate in *ori*λ-dependent Replication Initiation

The severity of the effect of P expression on host cell viability depends on its level of expression. When *P* is expressed from a λ phage during the lytic cycle, P protein is produced at a level sufficient for a few rounds of θ replication, but this low level of P is not lethal to cells (Ogawa and Tomizawa, 1968). How the level of P is held down when the cell may contain dozens of unpackaged λ genomes remains a mystery. λdv plasmids express both *O* and *P* at levels greater than those present in replicating phages. However, host cells can stably maintain these plasmids with no apparent loss of viability or slowing of growth rates (Ogawa and Tomizawa, 1968). In contrast, *O* and *P*, hugely over-expressed from tandem p_L and p_R promoters (Tsuriomoto *et al,* 1982), or *P* expressed alone (Klinkert and Klein, 1979), inhibit host cell DNA synthesis and cell growth, eventually leading to significant cell death. How can these incongruities be explained?

Essentially, the first difference is gene dosage; small amounts of P can be tolerated by cells. The results by Klinkert and Klein (1979) suggested that fairly high levels of P protein, expressed from a *lac* promoter, can inhibit cellular DNA initiation. However, Klinkert and Klein (1979) also demonstrated that P protein caused cellular filamentation, which they attributed to P-mediated induction of the SOS response. As previously mentioned, the SOS response is only induced when DNA propagation is inhibited. Thus, these results, in accordance with the results obtained by myself, suggest that high levels of P protein inhibit both the initiation and elongation stages of host cell DNA replication. I propose that in low concentrations, P can dissociate the

DnaB-DnaC complex, inhibiting host cell DNA replication initiation, as previously described. I also propose that in higher concentrations, P can bind to and inactivate DnaB that is actively unwinding DNA in a propagating replication fork, thus inhibiting the elongation stage of cellular DNA replication, a process which induces the SOS response.

I propose that the severity of P-Interference depends on whether or not *ori*λ-dependent replication initiation can proceed from the plasmid construct. It has been shown that P-mediated cell killing is less severe when *P* is co-expressed with *O* (e.g. λdv plasmids). In these situations, the over-produced *O* and *P* gene products are able to function in *ori*λ-dependent replication initiation, leading to the formation of the *ori*λ-O-P-DnaB complex. The host heat shock complex, DnaJ, DnaK and GrpE, recognize and bind to the P-DnaB components of this complex and remove P. DnaB unwinds the λ replication fork, and is then released, and is now free to function in a new round of DNA replication, either cellular or phage, depending on whether it binds to DnaC or P. The heat shock proteins have been proposed to alter the structure of P during its disassembly from the *ori*λ-O-P-DnaB complex (Hoffmann *et al,* 1992), so that it becomes incapable of binding to DnaB. Thus, the heat shock proteins, in essence, play an important role in "de-toxifying" the P protein. However, when *P* is expressed in the absence of *O* and *ori*λ, the free floating P-DnaB complex is not recognized by the heat shock proteins, and DnaB remains in a dead-end complex with P (Liberek *et al,* 1988; Zylicz *et al,* 1989). In this situation, the cell must rely on the ClpXP protease to remove the P protein. Whether ClpXP recognizes free P or P bound to DnaB is unknown. In contrast, when P is able to function in phage DNA

replication, the cell can rely on two independent mechanisms for "de-toxifying" P – the heat shock proteins and ClpXP.

4.1.5. P-Interference Conclusions and Further Considerations

I have demonstrated that: i) λ *P*, expressed from the plasmid pHB30 at 42°C, kills approximately 65% of host cells. ii) The remaining 35% are shown to be induced for SOS-mediated cellular filamentation. iii) The plasmid-mediated induction of the SOS response is specific to P, as plasmid pHB31, containing an in-frame *P* deletion, was unable to produce filamented host cells. iv) P-mediated cell killing is enhanced in cells defective for ClpXP, suggesting that this protease plays a role in the degradation of accumulated P protein. v) Host cells containing the *grpD*55 allele of *dnaB* remain sensitive to P-Interference. vi) High levels of P protein, expressed from the plasmid pHB30, interfere with the replication from a cryptic λ prophage. vii) Mutant pHB30 plasmids, able to produce stable transformants at 42°C, contain true reversions of the *cI*[ts]857 point mutation.

I propose that high levels of P protein, unable to participate in *ori*λ-dependent replication initiation, accumulate in host cells and inhibit host cell DNA replication. I propose that moderate levels of P protein will primarily inhibit host cell DNA replication initiation, but as P levels increase, the elongation stage will also be inhibited, which induces the SOS response. The inhibition of cellular DNA replication is ultimately lethal if *P* expression is not turned off. I propose that the term "P-killing or P-lethality" be changed to "P-Interference" to highlight the finding that P protein does not primarily kill host cells; it interferes with host cell DNA replication,

which may eventually be lethal.

I also propose that P interferes with host cell DNA replication via its role in sequestering DnaB, and possibly of DnaA. While it has been shown that P can disrupt a pre-formed DnaB-DnaC complex, it is not known whether P can disrupt a preformed *ori*C-DnaA-DnaB complex. If P is capable of dissociating such a complex, this may explain the ability of mutations in DnaA to suppress π mutation of P.

I suggest that if P protein is able to participate in phage λ replication initiation, higher levels of P can be tolerated by host cells. It has been suggested that interaction with the host heat shock proteins DnaJ, DnaK and GrpE, leave P in an altered conformation, unable to bind to DnaB. In essence, the heat shock proteins "de-toxify" P. Thus I propose that *E. coli* has two independent systems to "de-toxify" λ P protein. If the heat shock proteins are unable to inactivate P, ClpXP is incapable of degrading all of the potentially toxic P remaining in the cell, leading to the P-Interference phenotype.

The plasmid system that I have used, pHB30, expresses several proteins at 42°C (*i.e.* Cro-O fusion, P and Ren). Construction of a plasmid in which *cro-O* and *ren* have been removed will enable us to definitively measure the effect of P protein, without the added uncertainty caused by the presence of other co-expressed proteins. While I have used genetic evidence to propose that P is degraded by the ClpXP protease, biochemical assays must be done in order to directly demonstrate this. It is not known whether ClpXP can bind to free P, P-DnaB or P after it has been altered by the heat shock proteins. Undertaking these biochemical studies will greatly enhance the understanding of P-Interference. Further study must be done to elucidate the

relevance of the interaction between P and DnaA, to understand if this interaction has a role in phage λ replication, or if it is an anomaly specific to λdv replication. The finding that a single point mutation in the C-terminal region of *P* can suppress many different host defects is intriguing. NMR or X-ray crystallography structures of the P protein and the π *P* mutants interacting with DnaB would greatly aid our understanding of how P functions. One problem requiring solution is which one of DnaB's several conformational states does P bind to? Can P interact with DnaB and/or DnaA bound to DNA? As well, a crystal structure of P would be able to tell us about the supposed conformational change imposed by the heat shock proteins, which would greatly enhance our understanding of mechanism(s) of P action, both in λ replication initiation and P-Interference.

4.2. INHIBITION PHENOTYPE SPECIFIC TO *rep*λ PHAGE DEVELOPMENT

4.2.1. λdv Plasmids Exhibit the IP

As previously described, λdv plasmids are capable of autonomous replication, dependent on O and P activity at *ori*λ. λdv plasmids, originally derived from the virulent phage λvir, contain the λ genes r*exB, rexA, cI, cro, cII, O, P* and *ren*. The presence of the v1v3 operator mutations prevents CI from repressing transcription from p_R. Thus, in λdv plasmids, p_R transcription is suggested to be solely regulated by Cro activity (Matsubara and Kaiser, 1968; Matsubara, 1976). Early studies with λdv constructs suggested that they exhibited λ immunity (even though CI cannot regulate

p_R transcription of λdv DNA, it should still be able to bind to the operators of infecting phage). However, it was noted that there was something unusual about λ immunity expressed from λdv plasmids (Matsubara and Kaiser, 1968; Kumar and Szybalski, 1970). In normal situations, λ immunity is specifically directed towards homo-immune phage, *i.e.* phage carrying the λ immunity region (O_L-*rexB*-*rexA*-*cI*-O_R); however, hetero-immune (e.g. λ*imm*434 or λ*imm*21) or virulent phage (e.g. λvir) retain the ability to develop, as the CI made by *imm*λ phage cannot bind to the distinct operator sites of these phages. In contrast, host cells containing λdv plasmids inhibited *imm*λ phage (as expected), but also partially inhibited λ*imm*434 and λvir development. λ*imm*21 and λ*imm*80 phage were able to plate without difficulty (Matsubara and Kaiser, 1968; Kumar and Szybalski, 1970). The ability of λdv plasmids to inhibit λvir development was rationalized by the suggestion that λdv plasmids must make more CI repressor than a λ prophage, and that the higher levels of repressor would eventually bind the λvir operators (Matsubara and Kaiser, 1968). However, CI levels were not actually measured. No explanation was given to explain the inhibition of λimm434 development.

When actual RNA transcription levels from λdv plasmids were measured, it was demonstrated that very little (Kumar and Szybalski, 1970) or no (Hayes *et al,* 1997) *cI* was actually transcribed. These results showed that the inhibition of super-infecting phage development by λdv plasmids was not due to CI repressor activity. It was suggested that the λdv-mediated inhibition of phage development might be due to competition between the plasmid and super-infecting phage for the bacterial transcriptional and/or replication apparatus (Kumar and Szybalski, 1970). These

workers proposed that the competition must involve a site that is unique in the λ*imm*21 and λ*imm*80 phages, as they are able to escape inhibition (Kumar and Szybalski, 1970); however, no further work was undertaken in this area.

In 1978, Rao and Rogers demonstrated that cells containing a pBR322/λ hybrid containing the λ genes *N* through *cII* plus the N-terminal region of gene *O*, including the origin up to the *Eco*RI site in the AT rich region, inhibited the plating of λvir and λ*imm*434 infecting phage, but allowed λ*imm*21 to plate at high efficiency (Rao and Rogers, 1978). They reported isolating mutants of λvir and λ*imm*434 which were capable of plating on these cells at high efficiency, but these mutants were not characterized any further.

It appears that the general plasmid-dependent phage exclusion phenomena I have described herein as IP was noted by three different research groups over the past 35 years; however, the mechanism for CI-independent inhibition of super-infecting phage development by λ plasmids remained uncharacterized.

4.2.2. The Non-Immune Exclusion Phenotype

It is important to differentiate the IP, described here, from another phage exclusion system that was described and termed Non-Immune Exclusion (Nie) Phenotype (Hayes and Hayes, 1986; Hayes *et al,* 1998). Cells containing a ts cryptic λ*cro*$^{-}$ prophage were shifted to 42°C, resulting in prophage induction and replication and subsequent host cell killing, *i.e. cis* replicative killing. It was found that a subset of cells surviving *cis* killing from the induced cryptic prophage had acquired a new phenotype, which was termed Nie. At 30°C, the survivor clones were able to inhibit

the plating of *imm*λ phage, *i.e.* homo-immunity. However, the Nie Phenotype allowed the survivor clones to inhibit the plating of *imm*λ AND *imm*434 phage at 42°C (Hayes and Hayes, 1986). The Nie clones had also lost the ability to exclude T4*rII* phage at 42°C, suggesting that *rexA-rexB* transcription had been perturbed (Hayes *et al,* 1998). It was demonstrated that the Nie clones contained IS2 insertion elements or multiple point mutations within genes *O* or *P*, which prevented initiation of prophage replication (Hayes *et al,* 1998); because the prophage was unable to initiate replication, the cells survived at 42°C. The Nie exclusion-state was attributed to the constitutive expression of the defective λ fragment in the survivor cells, made possible by the acquired replication defect(s) (Hayes *et al,* 1998). λvir phage and spontaneous *imm*λ mutants were able to plate on Nie survivor cells at 42°C. The spontaneous *imm*λ mutants were called λse (suppress exclusion), and they were shown to contain mutations that mapped to three sites within o_R (Hayes and Hayes, 1986). The o_R mutations prevented lysogenization by interrupting CI repressor transcription from p_{RM}, and made the phage insensitive to replicative inhibition, meaning they were able to replicate independently of CI activity (Hayes and Hayes, 1986).

As noted, cells exhibiting the temperature sensitive Nie Phenotype have altered immunity profiles. They exhibit normal λ immunity at 30°C, but inhibit *imm*λ, *imm*434 and T4*rII* phages at 42°C; however, λvir and λse mutants are able to plate on Nie cells at 42°C. While the exact mechanism of exclusion remains unclear, it was suggested (Slavcev and Hayes, 2005) to likely depend upon the anomalous expression of the *rexA* and *rexB* genes. In contrast, the IP, which will be described in detail below, inhibits *rep*λ phage development, likely at the stage of replication initiation.

While λvir and λse phages (*i.e.* λse100a, λse101b and λse109b) escape the Nie Phenotype, these phage remain sensitive to the IP; suggesting that the two exclusion systems are essentially different.

4.2.3. Summary of Important IP Findings

I have demonstrated: i) Plasmids containing the λ t_O-*oop*-p_O through *ori*λ DNA sequence inhibited the development of an infecting *rep*λ λ*cI*857 phage or the development of an induced *rep*λ λ*cI*857 prophage. ii) The *rep*P22 phage λ*cI*857(*18,12*)P22, was insensitive to plasmids containing the λ t_O-*oop*-p_O through *ori*λ DNA sequence. Sequence analysis revealed that this hybrid phage contained *imm*λ, a hybrid λ/P22 *oop* gene, and P22 genes *orf48, 18 and 12*. iii) *Rep*λ phages containing *cI* or o_R defects or CI-independent rightward promoters remained sensitive to the IP. iv) The *rep*λ phages λ*imm*434*cI*$^-$, λvir and λ *imm*21*cI*$^-$ were able to partially escape the plasmid-mediated inhibition of growth; they were all able to plate with relatively high efficiency, but plaque sizes (indicative of phage burst) were greatly inhibited; with λ*imm*434*cI*$^-$ remaining the most sensitive and λ*imm*21*cI*$^-$ being least sensitive. v) Sequencing data revealed that λ*imm*434*cI*$^-$ and λvir retained wildtype λ sequence from *ice* through *ori*λ, thus the reason for their partial escape from the IP remains unknown; λ*imm*21*cI*$^-$ was shown to contain a hybrid 21/λ *oop* gene and a point mutation within the N-terminal region of gene *O*. vi) Ten independent λ*cI*857 mutants insensitive to plasmids containing the λ t_O-*oop*-p_O through *ori*λ DNA sequence were isolated. vii) None of the SIP isolates contained any DNA sequence alteration within the t_O-*oop*-p_O or *ori*λ regions. Three SIP isolates were shown to contain point mutations within the

N-terminal region of gene *O* (upstream of *ori*λ). Two SIP isolates were shown to contain point mutations near *cro*; one in the SD for the *cro* gene, and one within the coding sequence of *cro*. viii) All ten SIP lysogens were ampicillin resistant, suggesting that they had homologously recombined at least part of the pHB27R plasmid within their genomes. ix) PCR analysis was unable to confirm the presence of the pHB27R plasmid. x) Genetic assays were unable to confirm SIP phage replication from a pHB27R-derived ColE1 origin. xi) Plasmids in which 45 bp of the *oop* coding sequence was replaced were unable to confer the IP. xii) Plasmids deleted for the AT rich region of *ori*λ were fully functional in the IP. xiii) Plasmids deleted for all four iterons were unable to confer the IP. xiv) Plasmids deleted for iterons 3 and 4 were partially functional for the IP. xv) Phages blocked for *ori*λ-dependent θ replication, by the presence of the *dnaB*grpD55 allele, could bypass the *ori*λ block in multiply infected cells. xvi) The IP was bypassed in multiply infected cells. xvii) Cells expressing OOP RNA from a plasmid had decreased survivor frequencies (compared to cells without a plasmid) after λ*cI*857 infection at 30°C. xviii) Cells carrying multiple O-binding sites (*i.e.* iterons) from plasmids carrying *ori*λ had higher survivor frequencies (compared to cells without a plasmid) after λ*cI*857 infection at 30°C.

4.2.4. What We Think We Learned About the IP

It appears that the IP is directed towards *rep*λ phage; all tested *rep*λ phage showed at least some degree of plasmid-mediated inhibition of phage growth. The *imm*λ:*rep*P22 phage was completely insensitive to the IP. Earlier λdv studies had demonstrated that *rep*Φ80 phage were also insensitive to the λdv plasmid-mediated

inhibition of phage development (Matsubara and Kaiser, 1968; Kumar and Szybalski, 1970). These findings, combined with my results, suggest that the plasmid-mediated IP is specifically directed towards phage containing the *rep*λ region, classically defined as *ori*λ-*O-P*. The ability of the λ*imm*21*cI*⁻ (a *rep*λ phage with a hybrid *oop* gene) to partially escape the IP suggests that the actual sequence of OOP RNA produced by the phage is an important target of the inhibitory plasmids. It is interesting to note that the only phage able to completely escape the IP (λ*cI*857(*18,12*)P22) also contains a hybrid *oop* gene. It is likely that the different *rep* region (*ori*P22-18-12) accounts for this escape, but it remains possible that the hybrid *oop* is also important. Based on the finding that the λ*imm*21*cI*⁻ phage is the *rep*λ phage most able to escape the IP, and that it is the only *rep*λ phage containing a hybrid *oop* gene, I suggest that the *oop* gene may be a functional component of the *rep* region, *i.e.* *rep*λ actually consists of t_O-*oop*-p_O-*ori*λ-O-P. The finding that the IP is specific towards *rep*λ phage suggests that the step in phage development that is being inhibited is the stage requiring the elements encoded by the *rep*λ region, *i.e. ori*λ-dependent θ replication initiation.

The IP can be bypassed in multiply infected cells, in a manner similar to the way in which an *ori*λ-dependent replication block can be bypassed in multiply infected cells (Klein *et al,* 1979; Sclafini and Wechsler, 1981; Hayes and Hayes, unpublished data; cited in Hayes *et al,* 2005). This finding strengthens the suggestion that the IP is directed towards the *ori*λ-dependent θ replication initiation stage of *rep*λ phage development.

Multiply infected cells are likely able to bypass an *ori*λ replication block via

recombination-mediated events between two phage genomes contained within the same cell. It has been proposed that double strand break repair recombination intermediates in *E. coli* may be capable of initiating and undergoing DNA replication (Motamedi *et al,* 1999; Kuzminov, 1999). It is also possible that the circularized λ genomes could be producing linear multimers, formed by the rolling circle type of plasmid replication dependent on the RecF recombination pathway (Biek and Cohen, 1986; Cohen and Clark, 1986; Silberstein and Cohen, 1987; Kusano *et al,* 1989). If a cell contains more than one circularized λ genome (as a result of θ replication or merely due to multiple phage infections), recombination between the monomers might produce an invading strand which could lead to σ-like replication, independent of *ori*λ (Enquist and Skalka, 1973). These models propose that *ori*λ replication can be bypassed by a combination of recombination and σ replication. Whatever the mechanism of the bypass may be, both my studies and earlier work strongly suggest that *rep*λ phage can bypass an *ori*λ-dependent replication block in multiply infected cells. The ability of multiply infected cells to bypass the IP implies that the IP is directed towards *ori*λ-dependent θ replication.

I have isolated mutants of the *rep*λ phage λ*cI*857 (*i.e.* SIP phages) which are able to escape the IP. None of the SIP phages contain mutations within the OOP or *ori*λ components, but five SIP isolates were each shown to contain single point mutations. Three of these five SIP mutants contained a point mutation within the N-terminal region of gene *O*, and one contained a point mutation in the SD site of gene *cro* and one contained a point mutation within the coding sequence for gene *cro.* Note: six of the SIP isolates remain to be sequenced from o_R/p_R through *ice*, thus it is

not known if they also contain point mutations that we are unaware of. All ten SIP phages were able to produce ampicillin resistant lysogens. This result suggested that they contained pHB27R plasmids integrated within their genomes, likely as a result of homologous recombination. I was however, unable to find any additional genetic evidence for this; the phages were not able to replicate from a ColE1 origin under conditions which should inhibit *ori*λ-dependent replication. Further characterization of these phage isolates must be done, to determine why they escape the IP. It remains to be determined if the point mutations allowed escape, or if the plasmid integration was responsible for the SIP property of these phages.

I have shown that the λ plasmids must express the 77 nt OOP RNA in order to confer the IP; transcription of a random 77 nt non-OOP RNA by p_O did not confer the IP. This suggests that the physical transcription event from p_O in itself was not required, other than as a means of producing OOP RNA. It had previously been suggested (Bull, 1995) that the act of p_O transcribing away from *ori*λ might produce a highly negatively supercoiled micro-domain between p_O and *ori*λ, leading to the opening up of the leftward replication fork. The data that I have presented here argues against that hypothesis, as it appears that OOP RNA itself was important for the IP, not simply transcription from p_O. I have also shown that deleting the AT rich region of *ori*λ, previously shown to inactivate the origin, did not interfere with the IP, although deletion of the O-binding sites (iterons) did perturb the IP. These results suggest that a functional origin (enabling competitive replication initiation) was not required for a plasmid to inhibit *rep*λ phage development, but that the ability to bind O protein was important. In summary, in order to inhibit *rep*λ phage development, a

plasmid must express OOP RNA and must contain the O binding iterons.

4.2.5. Models to Explain λ Plasmid-Mediated Inhibition of the *ori*λ-dependent θ Replication Initiation Stage of *rep*λ Phage Development – A Synergistic Effect of OOP RNA and *ori*λ

In 1982, Anderl and Klein suggested that if the ratio of *ori*λ DNA:O protein is increased, replication will be inhibited, due to titration of O protein. This idea led me to propose that midcopy pBR322/λ plasmids expressing OOP RNA and containing the *ori*λ iteron sites likely act as competitor origins, sequestering the O protein of incoming *rep*λ phage. I have demonstrated that active *ori*λ replication from the plasmid is not required; merely the O binding sites are needed for the plasmid to inhibit *rep*λ phage development. I have shown that plasmids containing *oriλ*, without the concomitant expression of OOP RNA, mildly inhibit the replication of incoming *rep*λ phage. The simultaneous presence of OOP RNA somehow enhances the competitiveness of the iteron containing plasmid for *rep*λ initiator proteins. The requirement of OOP RNA for the IP supports my proposal that OOP RNA is an important accessory component for some aspect of λ replication initiation.

4.2.5.1. Model I: OOP RNA Negatively Regulates Translation of *O* and *P* mRNA

It has been directly demonstrated that OOP RNA binds to *cII* mRNA, leading to its degradation (Krinke and Wulff, 1990a; Krinke and Wulff, 1990b) in an RNaseIII-dependent reaction. Experimental evidence suggests that OOP RNA, expressed from midcopy plasmids, interferes with the Rex Exclusion phenotype

(Hayes *et al,* 1997). Significant p_O/p_{Lit1} and OOP RNA/*rexB* mRNA sequence similarities are likely more than co-incidental (Horbay *et al,* in press). The sequence similarities, combined with the experimental evidence, suggest that OOP RNA plays a role in modulating *rexB* gene expression, and thus the Rex Exclusion Phenotype. DNA sequence analysis has revealed potential OOP RNA binding sites in the SD regions for the *O* and *P* genes (Horbay *et al,* in press). It is possible that OOP RNA might bind to these sites and regulate *O* and *P* translation.

I propose that the t_O-*oop*-p_O-*ori*λ plasmids inhibit *ori*λ-dependent θ replication initiation by acting as competitive origins for incoming *rep*λ phage initiator proteins O and P. The presence of the iterons alone sequesters some of the *rep*λ O and P proteins, but enough initiator proteins remain unbound to promote some phage development. I also propose that OOP RNA binds to the SD sites of the *O* and/or *P* genes, decreasing the amount of initiator proteins made by the infecting phage. Because there are so many potential OOP RNA binding sites in the λ genome (*i.e. cII, rexB, O* and *P* regions), the inhibition of initiator gene expression by OOP is slight; so by itself, OOP RNA has no discernible effect on *O* and *P* gene expression levels. Combining the two negative regulatory elements, OOP and *ori*λ, on a mid-copy plasmid, has a synergistic effect on *rep*λ phage development. OOP RNA decreases the amount of O and P proteins made, and the majority of the proteins that are made end up binding to the numerous plasmid iteron sites, effectively inhibiting *rep*λ phage development. This model is entirely hypothetical, and the presumptive ability of OOP RNA to regulate initiator gene expression remains to be shown experimentally. There are several well documented examples of antisense RNAs with multiple targets, thus it remains

feasible that OOP RNA could bind to several sites on the λ genome, regulating gene expression.

4.2.5.2. Model II: OOP RNA Antisense Regulation of *cII-O-P-ren* mRNA

In 1990, Krinke and Wulff (1990a) demonstrated that the OOP-*cII-O-P-ren* mRNA complex could somehow be cleaved in an RNaseIII-independent reaction. In contrast to the RNaseIII-dependent cleavage reaction, the RNaseIII-independent cleavage resulted in stability of the *cII* mRNA fragment and subsequent degradation of the *O-P-ren* mRNA fragment (Krinke and Wulff, 1990a). These findings were never studied in any further detail; however, they imply that OOP RNA binding to *cII* mRNA has a downstream effect on *O-P-ren* gene expression. In the presence of OOP RNA, *cII-O-P-ren* mRNA can be cleaved in either an RNase III-dependent or RNase III-independent manner.

In a second model to explain the role of OOP RNA in the IP, I propose that OOP RNA inhibits *O* and *P* gene expression by binding to *cII-O-P-ren* mRNA. If RNaseIII-independent cleavage of the DS RNA OOP-*cII-O-P-ren* mRNA complex occurs (in contrast to an RNase III-dependent cleavage event), further degradation of *O-P-ren* mRNA will occur, thus inhibiting the expression of genes *O* and *P*. In a manner similar to the one proposed above, OOP-mediated degradation of *O* and *P* mRNA is not very efficient, thus OOP RNA expressed alone does not have a discernible effect on *rep*λ phage development. When OOP and *ori*λ are combined on a midcopy plasmid, the synergistic effect described above is seen. *O* and *P* expression is decreased by OOP regulation, and any O and P proteins that are made bind to the

plasmid origins and are unavailable for phage replication, effectively inhibiting *rep*λ phage development. The mechanism responsible for the observed OOP-mediated, RNaseIII-independent degradation of *O-P-ren* mRNA has yet to be determined.

4.2.6. Hypothetical Role for OOP RNA in λ Replication Initiation

If OOP RNA is shown to be capable of regulating *O* and/or *P* gene expression, a model can be proposed for the involvement of OOP RNA in the regulation of λ replication initiation. OOP RNA expression is conveniently regulated so that there are high levels of OOP RNA near the end of the θ mode of replication from *ori*λ, which is dependent upon *O* and *P* gene expression. While θ replication begins within two minutes of phage infection, OOP RNA levels do not begin to increase until seven to nine minutes after infection (Hayes and Hayes, 1978), reaching a maximum level shortly before switch to the σ mode of replication that occurs 15 minutes after infection. The switch from θ to σ has been demonstrated to occur precisely 15 minutes after infection, suggesting that the switch itself must be tightly regulated. Only a few rounds of θ replication are thought to occur before the switch to the σ mode of replication, which is believed to be O and P-independent. It has been shown that the inactivation of ts O or P proteins can immediately shunt the phage into the σ mode of replication (Takahashi, 1975a; Klinkert and Klein, 1978; Erdile and Inman, 1984). I suggest that the switch from θ to σ depends on actively shutting down the θ mode of replication by inhibiting the expression of the initiator O and P proteins. I propose that OOP RNA plays a natural role in regulating *O* and *P* gene expression levels during phage λ development; subsequently regulating the shift from the θ to the

σ mode of replication, *i.e.* once OOP levels reach a level where *O* and *P* expression can be perturbed (approximately 15 minutes after infection), the phage is shunted into the σ mode of replication. It has been suggested that DnaA plays a similar role in the replication shift via the regulation of P activity. This may be the reason why OOP RNA has not been unequivocally identified as a component of the λ replicator. In the absence of OOP RNA, DnaA may still be able to regulate the shift from θ to σ.

4.2.7. IP Conclusions and Future Considerations

My data suggests that plasmids expressing OOP RNA and containing the iterons from *ori*λ inhibit the *ori*λ-dependent θ replication of *rep*λ phage. The *ori*λ-replication block can be bypassed in multiply infected cells, likely via recombination-mediated processes.

I propose that OOP RNA and *ori*λ, present together on a midcopy plasmid, act synergistically to inhibit the *ori*λ-dependent θ replication initiation stage of *rep*λ phage development. I propose that OOP RNA interferes with the expression of *O* and *P*, decreasing the amount of O and P available for *rep*λ phage replication, and that any initiator proteins that are produced bind to the iterons from the multicopy plasmids, and are thus unavailable for *rep*λ phage replication.

While I have proposed that the IP is inhibiting *ori*λ-dependent θ replication, direct DNA replication studies must be done to definitively prove or disprove this hypothesis.

I have isolated phage mutants able to escape the IP; further characterization of these mutants will help to elucidate the mechanism of the IP, particularly in

understanding the phage targets of the plasmid-mediated inhibition.

This study has made it apparent that OOP RNA does play a role in the regulation of *ori*λ-dependent replication. Further studies must be done to unequivocally demonstrate the mechanism of OOP RNA action. OOP RNA binding studies must be done to determine whether OOP is capable of binding to the proposed sites on the λ genome. If OOP binding does occur, then further studies can be done to determine the effect of the binding, *i.e.* if OOP binding affects gene expression and subsequent DNA replication. It has been demonstrated that some antisense RNA molecules utilize an accessory protein to aid in antisense regulation. Experiments to determine if OOP uses a helper protein would aid in elucidating its mechanism of action.

4.3. OVERALL CONCLUSIONS

Bacteriophage λ has been used as a model replicon for decades. Even after all of this time, λ continues to surprise us with the depths of its genetic complexity. We have used λ replication as a basis for understanding more complicated (*i.e.* eukaryotic) systems; however, it turns out that there are still many things regarding the fine-tuning aspects of phage replication that we do not know. To this day, we still do not understand transcriptional activation, the requirements for bi-directional θ replication or the mechanism of the switch from the θ to the σ mode of replication. Ideas and theories abound, but are yet to be unequivocally proven experimentally. Many of the things we think we know regarding λ replication have been demonstrated *in vitro*, using high concentrations of proteins. The validity or *in vivo* relevance of these

studies remains questionable. The use of λdv plasmids as a source of template λ DNA in these studies may also be a problem, as it has been demonstrated that these plasmids do not replicate in exactly the same manner as phage λ does. While *in vivo* studies are more complicated and the results more difficult to interpret, using such systems may be the only way we will ever truly understand bacteriophage λ DNA replication.

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